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**The effects of blueberry and blackcurrant powder addition to oat
bran paste on its physicochemical characteristics, and their
subsequent *in vitro* glycaemic response, and cellular anticancer
properties.**

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy

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by
Xiaodan Hui

Lincoln University
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Abstract of a thesis submitted in partial fulfilment of the
requirements for the Degree of Doctor of Philosophy.

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Cereal foods supplemented with fruit ingredients have attracted the attention of consumers due to their richness of bioactive compounds (phenolic compounds), and potential health promoting effects (reducing the risk of obesity, type 2 diabetes, and cancers). Despite the well-known health benefits of the purified ingredients from berry fruits, few studies have reported the actual use of these ingredients in relation to the nutritional effects of a food matrix and the consumption of cereal foods supplemented with berry fruits. Although using simple purified ingredients may help to identify mechanisms of action, the additive or synergistic effects provided by a food matrix cannot be observed by not including complex food systems, since the functionality of foods is highly influenced by the interactions between all of the components within the food matrix.

Oat bran was enriched with 0%, 10%, 15% and 25% (w/w) of blueberry and blackcurrant powder, respectively. The Rapid Viscosity Analyser (RVA) equipment was used to form oat bran pastes which were enriched with blueberry or blackcurrant powder in order to study the effects of the food matrix on physicochemical characteristics, nutritional and functional properties of the pastes. High performance liquid chromatography (HPLC), and an *in vitro* digestion process, were performed to observe the effects of bioactive compounds from

blueberry and blackcurrant powders on the predicted *in vitro* glycaemic response. Results showed that the oat bran paste containing blueberry powder, or blackcurrant powder, increased the values for total phenolic compounds, and the antioxidant capacity. However, the total monomeric anthocyanin content decreased after the *in vitro* digestion. Cyanidin, delphinidin, malvidin, pelargonidin, and peonidin in the extracts of blueberry and blackcurrant enriched pastes were identified by the pH differential method. The extent of starch degradation, and the area under the curve (AUC) of reducing sugar released, of blueberry and blackcurrant enriched pastes decreased during the *in vitro* digestion.

The *in vitro* α -amylase and α -glucosidase inhibitory activities of the extracts from blueberry and blackcurrant enriched oat bran pastes were determined, and the potential mechanisms of the interaction of anthocyanins on digestive enzymes were studied. Addition of blueberry or blackcurrant powder into oat bran paste significantly increased the α -amylase and α -glucosidase inhibitory activities and decreased IC₅₀ values of the pastes ($p < 0.01$). The extracts of blueberry and blackcurrant enriched pastes were observed to be mixed-type inhibitors against α -amylase, while they were competitive inhibitors towards α -glucosidase. In addition, their enzymes inhibitory activities were hypothesised to be driven by hydrogen bonding. Cyanidin-3-glucoside and delphinidin-3-glucoside in the extracts of blueberry and blackcurrant enriched pastes had stronger binding affinities compared to malvidin-3-glucoside and cyanidin-3-rutinside.

The anticancer bioactivity towards HepG2 cell line between the extracts of undigested and intestinal digested blueberry and blackcurrant enriched pastes were compared, and the potential mechanisms on their anticancer properties were also studied. The extracts of undigested blueberry and blackcurrant enriched pastes inhibited the cell growth, and cell invasion, towards HepG2, more significantly than the extracts of digested pastes ($p < 0.01$). Mechanistic studies suggested that the extracts of blueberry and blackcurrant enriched pastes

induced HepG2 cell apoptosis by initiation of the cell cycle arrest and regulation of the expression of apoptotic-related proteins, including Bcl-2, Bax, and caspase-3. The intracellular reactive oxygen species level of extracts treated-HepG2 cells increased significantly ($p < 0.01$) via regulation of the nuclear factor-like 2 (Nrf2)/heme oxygenase 1 (HO1) signalling pathway. The synergistic effects of this study suggest that enrichment of oat bran with blueberry and blackcurrant powder has the potential to develop a range of functional foods, and there may be a new and effective option to prevent and control chronic diseases in human.

Keywords: glycaemic response, *in vitro* digestion, phenolic compound; anthocyanidin, antioxidant, type 2 diabetes, apoptosis, anti-proliferation, invasion, reactive oxygen species ROS, anticancer, HepG2, α -amylase, α -glucosidase, molecular docking, enzyme kinetics

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Chapter 1

Introduction and thesis outline

1.1 Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disease characterised by chronic hyperglycaemia due to insulin resistance and/or insufficient insulin production by the pancreatic β -cells (insulin insufficiency) (American Diabetes, 2013; Toniolo *et al.*, 2019). Pharmacologic interventions are costly, and often associated with adverse side effects, while nutritional therapy remains central to the prevention, and treatment of T2DM (Sanders, Monogue, Jodlowski, & Cutrell, 2020). Overweight and obese status are often associated with an increased risk for many cancer types, including liver cancer, breast cancer (in women past menopause) colon and rectal cancer, and endometrial cancer (Pi-Sunyer, 2009). Most of the molecules that are being investigated as potential mediators between obesity and cancer are themselves cancer-promoting at high concentrations, and can induce growth and proliferation of malignant cells (Stone, McPherson, & Gail Darlington, 2018).

Lifestyle and dietary habits are major factors in determining the development and progression of T2DM (Kolb & Martin, 2017). Evidence has shown that dietary patterns, including regular consumption of plant-based foods rich in phytochemicals, may confer specific molecular and cellular protection in addition to the overall epidemiologically observed benefits (lower rates of obesity and cancer risk), further enhancing health (Kristo, Klimis-Zacas, & Sikalidis, 2016; Ros & Hu, 2013). The health benefits of plant-based foods may be associated with bioactive compounds, among which are anthocyanins, which also have antioxidant properties. Anthocyanins are polyphenols that belong to the flavonoid subgroup and they are the natural dark pigment colour in plant foods (Di Gioia *et al.*, 2020; Panche, Diwan, & Chandra, 2016).

Phenolic compounds are abundant in berry fruits and cereal foods, such as blueberry, blackcurrant, and oat bran (including avenanthramids that are unique to oats) (Soycan *et al.*, 2019). Studies have reported beneficial effects of berries, or their isolated purified constituents, on cancer treatment, including the scavenging of free radical species, control of cell cycle progression, inhibition of cell proliferation, induction of apoptosis, inhibition of the activity of oncogenes, and inhibition of hormone or growth factor activity (Koolaji *et al.*, 2020; Kristo *et al.*, 2016).

Blueberries (*Vaccinium*) and blackcurrants (*Ribes nigrum*) are regarded as popular fruits that have gained the interest of the public and scientific communities due to their potential role in maintaining and improving health (Khoo, Azlan, Tang, & Lim, 2017). The scientific evidence (Tsuda, 2016) supporting the anti-diabetic and anticancer health benefits of blueberries and blackcurrants is growing. Several studies have found improvements in insulin resistance and glucose tolerance after the consumption of blueberries and blackcurrants in obese and insulin resistance rodents and humans (Calvano *et al.*, 2019; Esposito *et al.*, 2015; Nolan, Brett, Strauss, Stewart, & Shepherd, 2020). The role of breakfast cereals in a balanced healthy diet has been recognised for many years, particularly breakfast cereals which are rich in whole grain, or high in cereal fibre (Laskowski, Górska-Warsewicz, Rejman, Czacotko, & Zwolińska, 2019). Dietary guidelines illustrate that the high nutrient density of breakfast cereals is an essential source of key nutrients (Nicklas, Drewnowski, & O'Neil, 2014). Oat bran, normally consumed as ready-to-eat breakfast, forms part of well-balanced and nutritious diets (Rasane, Jha, Sabikhi, Kumar, & Unnikrishnan, 2015). In addition to being an important source of vitamins, fibre (especially β -glucan), and minerals, oat bran also contains potentially important sources of antioxidants and phytoestrogens. Research (Ulmius, Johansson-Persson, Krogh, Olsson, & Onning, 2011) has also revealed that oat bran may have positive effects on postprandial glucose levels, serum cholesterol and body mass index.

Berries have a positive image amongst consumers, and their inclusion in cereal products seems to have potential health benefits (Slavin & Lloyd, 2012). Some studies have focused on the antioxidant effects of single purified ingredients from bioactive compounds, such as the ferulic acid and anthocyanins from blueberry (Durazzo, 2017; Lourenço, Moldão-Martins, & Alves, 2019; Tenore, Novellino, & Basile, 2012). However, few studies have reported the actual use of the whole berry fruits in a food matrix, or even the effects of the whole food matrix when consumption of cereal foods which have been supplemented with berry fruits on physicochemical characteristics of foods, or functional properties on human nutrition. Since the functionality of foods is highly influenced by the interactions between food components, studies performed on pure components do not reflect a realistic *in vivo* situation. It is essential to understand the influence of the processing and the interactions between food components on the bioavailability of phenolic compounds.

Therefore, this study aims to evaluate the effects of enrichment with blueberry and blackcurrant powder on the physicochemical, anti-diabetic, and anticancer properties of the oat bran pastes.

1.2 Objectives

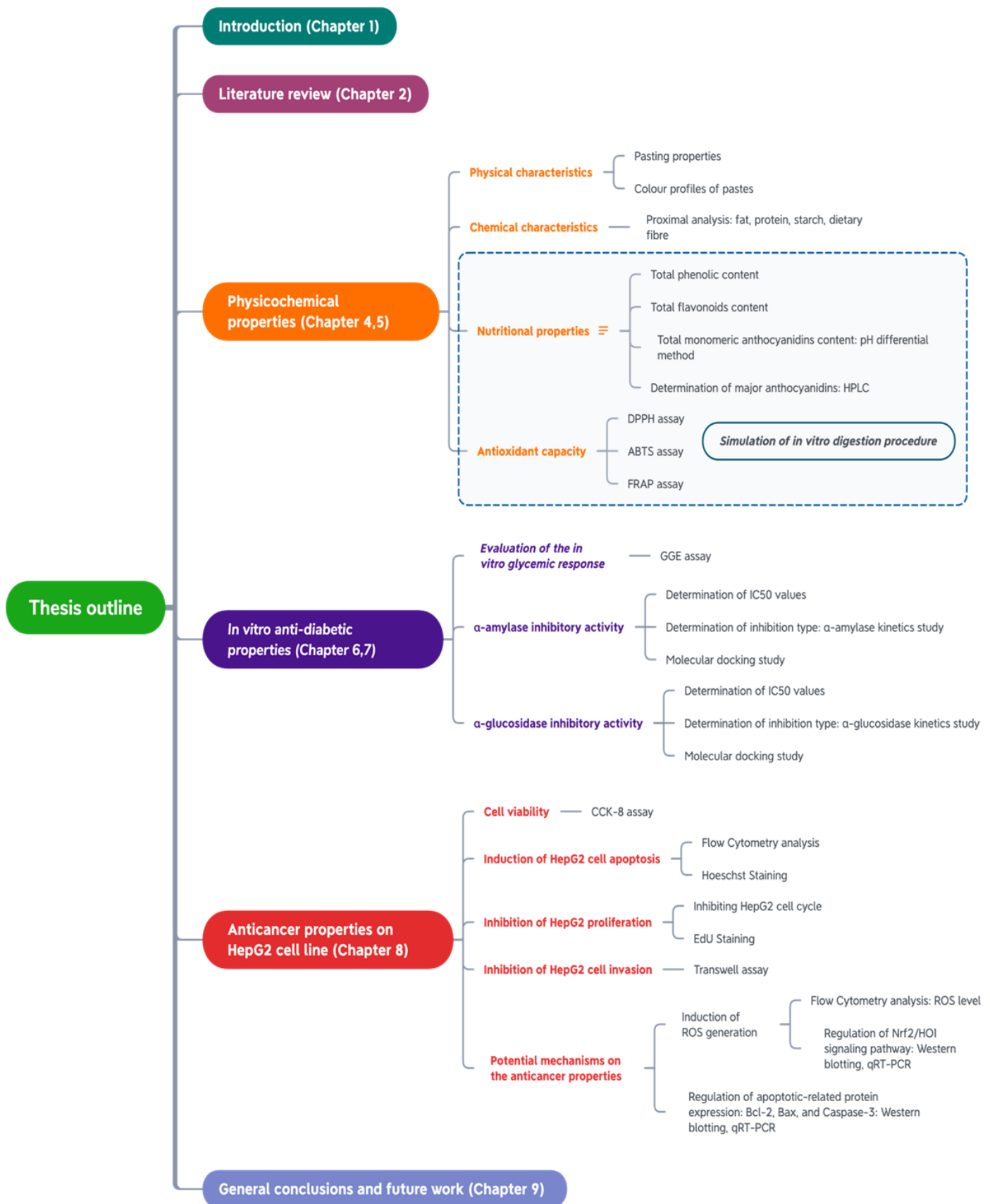
In order to achieve the aim, the following objectives were designed:

1. Investigation of the interaction of the polyphenols from blueberry and blackcurrant powder with other food components present in the food matrix.
2. Evaluation of antioxidant and glycaemic response of the blueberry and blackcurrant powder enriched pastes via *in vitro* analysis.
3. Evaluation of the anticancer properties towards HepG2 cell model of the blueberry and blackcurrant powder enriched pastes.

1.3 Hypotheses

1. Enrichment with blueberry or blackcurrant powder for oat bran pastes will increase the content of phenolic compounds and the antioxidant capacity of the pastes.
2. The phenolic compounds from blueberry and blackcurrant powder will reduce the reducing sugar released from oat bran paste during *in vitro* digestion, and this will involve inhibition of α -amylase and α -glucosidase activities.
3. Blueberry and blackcurrant powder enriched oat bran pastes will exert anticancer properties towards HepG2 cells, and this will involve inducing cell apoptosis, and inhibiting the cell proliferation and cell invasion of HepG2 cells.

1.4 Thesis outline



Chapter 2

Literature review

Abstract

Type 2 diabetes mellitus (T2DM) is a progressive metabolic disorder with hyperglycemia accompanied by abnormal carbohydrate, lipid, and protein metabolism. Insulin resistance, impairs insulin action, and also impairs β -cell function, both of which are key features of T2DM. T2DM can be treated, and many of its consequences avoided or delayed, with diet, physical activity, and medication. Understanding the potential mechanisms to alter the progression of the disease may create new insights into the prevention and treatment of T2DM. However, implementing the lifelong dietary changes needed for reducing weight is a challenging. Thus, there is a need for new food concepts that may contribute to dietary strategies used in preventing metabolic disorders. Berries, such as blueberries and blackcurrants, are well-known for containing large amounts of polyphenol compounds, which can exert beneficial effects in chronic diseases including metabolism disorders. Even though current evidence is promising (Stote *et al.*, 2020), more long-term, randomised, and placebo-controlled trials are needed to establish the role of blueberries and blackcurrants in preventing or delaying T2DM. In addition, firm conclusions regarding the anti-diabetic effect of blueberries and blackcurrants cannot be drawn due to the small number of clinical studies.

2.1 Introduction

There are now over 1.9 billion adult humans in the world who are overweight, over 600 million of these are obese, and this is accompanied by an increase in obesity-related disorders including type 2 diabetes Mellitus (T2DM) (Crane & McGowan, 2018). T2DM has created a huge burden on modern society and is a serious threat to human health. In 2014, 1.6 million deaths were caused by diabetes and an estimated 550 million people will be affected by it by 2060 (Lin *et al.*, 2018). T2DM is a progressive metabolic disorder with hyperglycemia accompanied by abnormal carbohydrate, lipid, and protein metabolism (Hameed *et al.*, 2015). However, insulin resistance defects in insulin action, and impaired β -cell function are key features in T2DM (Hameed *et al.*, 2015). In the current medical situation, with no effective treatment, recent works suggest that T2DM can be prevented by manipulation of lifestyle factors as an medication. Thus, understanding the potential mechanisms to alter the progression of the disease may create new insights into the prevention and treatment of T2DM (Colberg *et al.*, 2016).

Berries such as blueberries and blackcurrants are known to contain large amounts of polyphenol compounds, which can exert beneficial effects on chronic diseases including metabolism disorders (Khoo *et al.*, 2017). This review will summarise the current knowledge of pathogenesis of T2D (particularly insulin resistance), and the potential anti-diabetic effects of blueberry and blackcurrant.

2.2 Insulin resistance

Insulin is a major metabolic hormone secreted by pancreatic β -cells, and regulates the homeostasis of plasma glucose by inhibiting hepatic glucose output, increasing the glucose uptake rate of skeletal muscle and adipose tissue. Normally, insulin binds to its receptor on

the surface of the cell of peripheral tissues (Chadt & Al-Hasani, 2020; Qaid & Abdelrahman, 2016), including muscle, liver and adipose tissue, then activates various insulin signalling pathways via tyrosine phosphorylation of the insulin receptor substrate proteins (Boucher, Kleinridders, & Kahn, 2014). Insulin also promotes the intake of free fatty acids (FFA) by adipocytes in order to increase lipogenesis, in which the body's response in diabetic function of insulin is impaired (Sears & Perry, 2015). In insulin resistance subjects, the role of insulin glucose uptake is impaired, resulting in increased compensatory insulin secretion (Czech, 2017). An important indicator of insulin resistance is hyperinsulinemia, which is characterised by decreased sensitivity of tissues (primarily muscle) to insulin, and impaired utilisation of glucose (Di Pino & DeFronzo, 2019).

2.3 Blueberries and blackcurrants

Blueberries and blackcurrants are regarded as functional foods due to their biologically active compounds which provide clinically health benefits. Apart from vitamin C, folate and flavonoid, berries contain large amounts of phenolic compounds (Huang, Zhang, Liu, & Li, 2012; Skrovankova, Sumczynski, Mlcek, Jurikova, & Sochor, 2015). Studies have demonstrated that polyphenolic compounds can decrease the level of reactive oxygen species (ROS) (Olas, 2018; Oliveira, Carvalho, & Melo, 2014; Serino & Salazar, 2019). Inhibition of the lipid and protein oxidation has shown to be able to improve the quality of foods (Liu, Luo, & Wei, 2019; Yahfoufi, Alsadi, Jambi, & Matar, 2018).

Blueberries (*Ericaceous Vaccinium*) are an increasingly popular addition to Kiwi backyards (Khoo *et al.*, 2017). Recently, researches have illustrated that blueberries, and especially the flavonoids compounds present in the berry, such as catechin, epicatechin, and anthocyanins, can exhibit health benefits against degenerative diseases, such as T2DM, cancer and Alzheimer's disease (Kalt *et al.*, 2019; Kozłowska & Szostak-Węgierek, 2019). Blackcurrant

belongs to the *Ribes nigrum* group of *Grossulariaceae* plants. It is a rich source of naturally occurring anthocyanins (Zorița Diaconeasa, Loredana Leopold, Dumitrița Rugină, Huseyin Ayvaz, & Carmen Socaciu, 2015). Originating in Northern Europe and Asia, blackcurrants have been shown to have the potential to regulate and inhibit inflammation mechanisms (Bonarska-Kujawa, Cyboran, Żyłka, Oszmiański, & Kleszczyńska, 2014).

2.3.1 Flavonoids and anthocyanins in blueberries and blackcurrants

Flavonoid compounds in blueberry and blackcurrant fruits have a similar basic chemical structure of a common three-ring moiety (A-, C- and B-rings) with 15 carbon atoms (C6–C3–C6) (Amawi, Ashby, & Tiwari, 2017). The substitution of a functional group of the C-ring determines the classification of subclasses of flavonoids (**Fig. 2-2**). Flavonoids are divided into two main groups according to their structure, 2-phenylchromans (flavonoids) and 3-phenylchromans (isoflavonoids). The flavonoids group in berry fruits (**Fig. 2-1**) includes the subclasses of flavanones, flavones, flavonols, flavan-3-ols, and anthocyanidins, while the isoflavonoids includes the subclasses of isoflavones, isoflavans, and pterocarpanes (Silva *et al.*, 2020).

Anthocyanins are a major subclass of flavonoids, which are conjugated anthocyanidins. There are six main anthocyanidins, including cyanidin, malvidin, delphinidin, peonidin, petunidin, and pelargonidin. They are in the form of O-linked conjugates with numbers of sugars (Kalt, 2019; Zhang, Celli, & Brooks, 2019). The endogenous anthocyanins of blackcurrants and blueberries have shown in **Table 2-1** (Elks, Terrebonne, Ingram, & Stephens, 2015). The most common anthocyanidins found in blueberries are the 3-O-galactosides of cyanidin, delphinidin, petunidin and malvidin. Blackcurrants are characterised by the presence of the rutinosides and glucosides of delphinidin and cyanidin, in which the rutinosides being the most abundant, other anthocyanins in blackcurrants are present at much lower levels (Dangles & Fenger, 2018; Olivas-Aguirre *et al.*, 2016).

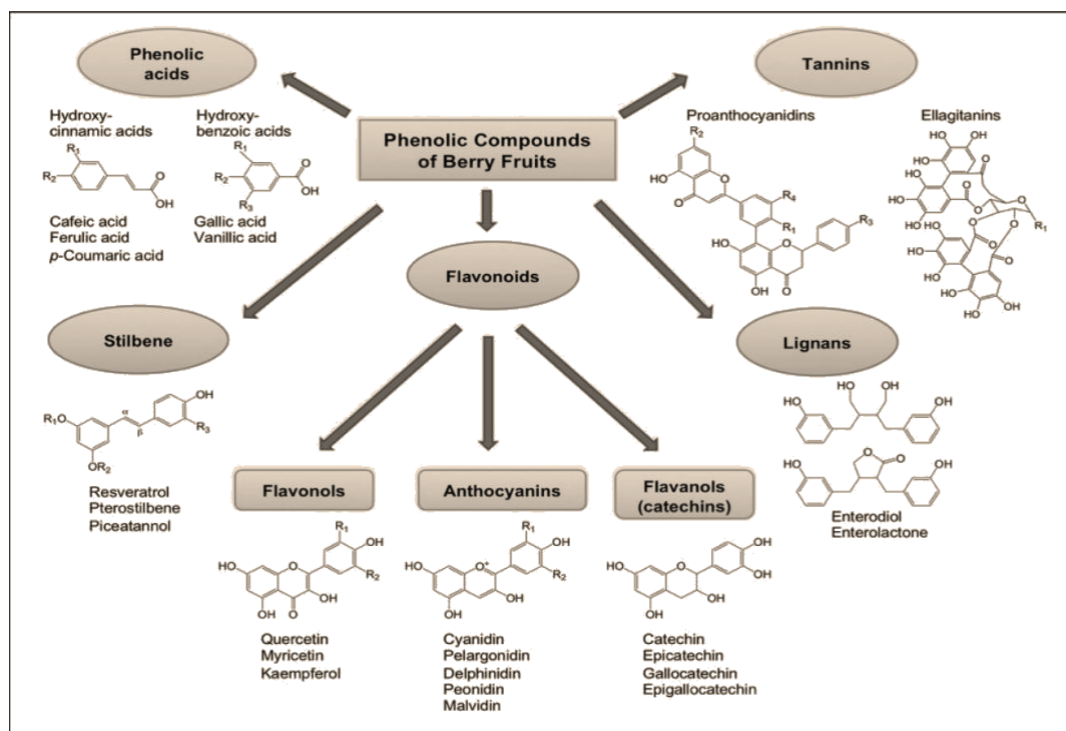


Figure 2.1 Phenolic compounds found in berry fruits

(Paredes-López, Cervantes-Ceja, Vigna-Pérez, & Hernández-Pérez, 2010)

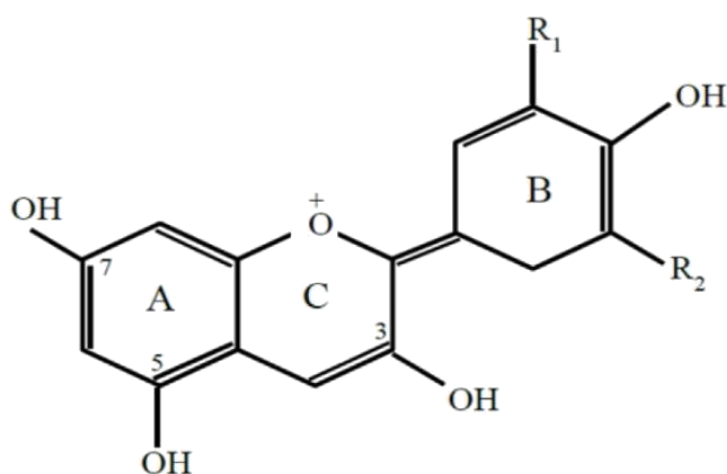


Figure 2.2 Chemical structures of major anthocyanins in berries

R1 and R2 can be H, OH or OCH₃ group (Kalt, 2019).

Table 2-1 The major anthocyanins present in blackcurrant and blueberry

Blackcurrant	R1	R2
Cyanidin	OH	H
Delphinidin	OH	OH
Malvidin	OCH ₃	OCH ₃
Pelargonidin	H	H
Peonidin	OCH ₃	H
Petunidin	OCH ₃	OH
Blueberry	R1	R2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH ₃	H
Petunidin	OCH ₃	OH
Malvidin	OCH ₃	OCH ₃

R1 and R2 (present in C-ring) are each independently H, OH or OCH₃ (Del Rio, Borges, & Crozier, 2010).

2.3.2 Bioavailability of phenolic compounds from blueberries and blackcurrants

Bioavailability has been defined as “the rate and extent to which the active ingredient or moiety is absorbed and becomes available at the site of action” (Menezes, Orlikova, Morceau, & Diederich, 2016; Thilakarathna & Rupasinghe, 2013). The bioavailability of polyphenols depends on the release of polyphenols from a complex, which, in turn, depends on the structure of the polyphenols, the complexity of the polyphenol-carbohydrate structure, and the possibility of enzymes to reach the carbohydrates (Van Hul & Cani, 2019). Flavonoid bioavailability is influenced by food matrix, absorption rate, metabolism and structure. Among the various flavonoids, isoflavones have the highest bioavailability, however, according to several researches, only a small fraction of anthocyanins are absorbed by humans (Williamson, Kay, & Crozier, 2018). In general, low molecular weight polyphenols are partially absorbed into

the body directly or after phase II enzyme-dependent metabolic conversion in small intestinal cells. (Cassidy & Minihane, 2017). After absorption from the small intestine, flavonoids reach the liver where they can be conjugated with bile components. As a result, no free flavonoid glycones can be found in plasma or urine, except for catechins (Thilakarathna & Rupasinghe, 2013). High molecular weight tannins and even low molecular weight polyphenols, such as flavonoids, are transported into the large intestine in their original form. They are then excreted with feces without intestinal absorption, or they are catabolized by enterobacteria (Williamson *et al.*, 2018). Interestingly, researchers have also proposed that the bioavailability of flavonoids depends on the variation between individuals since each subject has its own microbiota which may interfere with the bioavailability of anthocyanins (Eker *et al.*, 2019).

In vitro studies

The absorption of anthocyanins from blueberry extracts has been studied in the Caco-2 model (Kamiloglu, Capanoglu, Grootaert, & Van Camp, 2015). Liu *et al.* (2014) claimed that the transport efficiency of blueberry anthocyanin was low, 3-4% as the free hydroxyl groups and fewer OCH₃ groups in blueberry anthocyanin reduced its bioavailability.

Studies have examined the metabolic processes of anthocyanins involved in absorption. Correa-Betanzo *et al.* (2014) combined an *in vitro* gastrointestinal digestion process with a colon fermentation phase to assess the stability of blueberry anthocyanins and their metabolites. The recovery of polyphenol and anthocyanin was 94% and 97%, respectively, under the gastric digestion step. Following the intestinal digestion step the recovery of polyphenols and anthocyanins was reduced to 49% and 17%, respectively. The amounts recovered after the colon fermentation step were 42% and 1.5%, respectively. Another study (Levy, Okun, & Shpigelman, 2019) used LC-ESI-MS to determine the stability of the polyphenol components in blueberry extracts. The results showed that a significant decrease in the absorbance of the anthocyanin peaks was observed after the intestinal digestion step, which

indicated the anthocyanins experiencing ring cleavage since the pH shifted from an acidic environment (pH = 2) to an alkaline (pH = 8) environment. Only 50% of delphinidin- and malvidin-6-acetoyl 3-glucoside remained intact, while the other anthocyanins were degraded after the digestion process.

***In vivo* studies**

In human studies, analysis of anthocyanin concentrations using blood and urine is the most common method to understand the bioavailability of anthocyanins in humans. A recent study (Kuntz *et al.*, 2015) evaluated plasma and urine anthocyanin concentrations by UPLC–MS after consumption of blueberry juice/smoothie by 10 healthy subjects. Malvidin-3-glucoside, peonidin-3-glucoside, delphinidin-3-glucoside, cyanidin-3-glucoside, and petunidin-3-glucoside transformed into around 80% of the anthocyanins in the juice/smoothie. These compounds were detected in plasma and urine samples. There were no significant differences in the bioavailability of these anthocyanins in the juice or smoothie. However, the concentrations of peonidin-3-glucoside were higher in both plasma and urine than Malvidin-3-glucoside. Therefore, Kuntz *et al.* (2015) concluded that peonidin-3-glucoside is absorbed more rapidly in the circulatory system than malvidin-3-glucoside. Sandhu *et al.* (2016) reported that nonacylated anthocyanins were more bioavailable than acylated anthocyanins in freeze-dried strawberries. Only 0.002-0.003% of anthocyanins were detected in the human serum samples with the major anthocyanins being nonacylated (malvidin-3-galactoside, malvidin-3-glucoside and delphinidin-3-glucoside).

2.3.3 Potential adverse effects

The daily diet contains considerable amounts of flavonoids, most of which are considered safe (Panche *et al.*, 2016). One study (Bouayed & Bohn, 2010) reported that the intake of quercetin over a long time may cause mutagenicity and DNA damage, which may due to their pro-oxidant activity. The use of supplements, including non-nutritive supplements, has been on

the rise in recent years. Thus, the overuse of flavonoids should not be encouraged until their biological effects are proved.

2.4 Impact of food matrix on bioavailability of polyphenols in blueberries and blackcurrants

2.4.1 Processing effects

Understanding the effects of food processing on polyphenols and bioavailability is important since most of the food consumed on a daily basis is in a processed form (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013). Traditional methods of thermal processing, such as pasteurisation provide microbiological stability and extend shelf life (Deeth, 2017). However, these methods cause some undesirable changes such as degradation of polyphenols and other bioactive compounds. The possibility of ensuring food safety, and preserving bioactive compounds, has resulted in an increased interest in the minimal processing of foods using nonthermal methods, such as high-pressure processing and ultrasound (Putnik *et al.*, 2020). Studies (Marszałek, Woźniak, Skąpska, & Mitek, 2017; Nicolau-Lapeña *et al.*, 2019; Tsikrika, O'Brien, & Rai, 2019) have demonstrated that, in comparison with high-pressure processing, pasteurisation can cause greater degradation of polyphenols, anthocyanins, vitamin C, and the pigment of strawberry puree. Treatment with high-intensity ultrasound, resulted in the cavitation of cells, breaking down cell walls, and facilitated the extraction of bioactive compounds, thus increasing their bioavailability. Increased antioxidant capacity and monomeric anthocyanin content in red raspberry puree treated with high-intensity ultrasound were achieved by Golmohamadi, Möller, Powers, and Nindo (2013), when compare with those without the treatment with high-intensity ultrasound.

2.4.2 The interactions between polyphenols and the food components

Researchers have evaluated the influence of the food matrix composition on the bioavailability of anthocyanins (Igwe, Charlton, Probst, Kent, & Netzel, 2019). The concept of 'food matrix' points out that nutrients are contained in a larger continuous medium that may be of cellular origin (in fruits and vegetables), or a microstructure produced by processing, where they may interact at different length scales with the components and structures of the medium (Quirós-Sauceda *et al.*, 2017). However, data on how polyphenol bioavailability is affected by these components is scarce. It has been suggested that nutrients like proteins, carbohydrates and lipids that surround polyphenols inside the gastrointestinal tract, have a great impact on the bioaccessibility and bioavailability of polyphenols (Wojtunik-Kulesza *et al.*, 2020). Studies conducted in recent years have shown that nutrients protect polyphenols from oxidation during their passage through the gastrointestinal tract and deliver them to the colon more completely (Catalkaya *et al.*, 2020; Cueva, Silva, Pinillos, Bartolomé, & Moreno-Arribas, 2020; Kumar Singh *et al.*, 2019).

The interactions between polyphenols and lipids

Only a few studies have investigated the interactions between polyphenols and dietary lipids. Since most polyphenols are water soluble, dietary lipids are considered to have a limited influence on the uptake of polyphenols (Cory, Passarelli, Szeto, Tamez, & Mattei, 2018). Upadhyay and Dixit (2015) revealed that the use of polyphenols for creating functional foods could result in decreased oxidation of lipids and unsaturated fatty acids in foods, consequently reducing the intake of prooxidants. It was shown that lipids from foods could enhance acute absorption of some flavonoids, such as quercetin (Li *et al.*, 2016). Polyphenols can be absorbed with lipids, using the same metabolic pathways (D'Archivio, Filesì, Vari, Scazzocchio, & Masella, 2010). Therefore, it may be feasible to incorporate polyphenols in lipoprotein particles, such as high-density lipoprotein and low-density lipoprotein (Estrada-Luna *et al.*,

2018). However, the interactions of polyphenols with lipids, and its consequent effects in the body should be further investigated.

The interactions between polyphenols and proteins

Interactions of polyphenols with proteins have been shown to provide greater flavonoid stability over storage time, which potentially result from inhibiting the availability of auto-oxidative degradative reactions (Panche *et al.*, 2016). Proteins can bind to polyphenols mainly by non-covalent hydrophobic interactions (Shavandi *et al.*, 2018). Higher molecular weight polyphenols are able to bind more strongly to proteins than lower molecular weight polyphenols. The flexibility of the polyphenol molecule also plays an important role in these interactions (Sęczyk, Świeca, Kapusta, & Gawlik-Dziki, 2019). A previous study (Khoo *et al.*, 2017) reported that the ingestion of blueberries in association with milk impaired the *in vivo* antioxidant properties of blueberries and reduced the absorption of caffeic acid

The interactions between polyphenols and carbohydrates

In comparison to flavonoid-protein interactions, much less is known regarding the interaction between flavonoids and carbohydrates (Wojtunik-Kulesza *et al.*, 2020). Carbohydrates make the polyphenols bioaccessible in the colon after being exposed to enzymes and microorganisms (Tarahovsky, Kim, Yagolnik, & Muzafarov, 2014). Polysaccharides seem to form a water soluble “protein-polyphenol-polysaccharide” complex (Le Bourvellec & Renard, 2012), allowing them to perform the role of carrier for polyphenols through the gastrointestinal tract. One study (Etcheverry, Grusak, & Fleige, 2012) showed that in both rats and human subjects, phytic acid, a component of hulls of nuts, seeds and grain increased the bioavailability of blackcurrant anthocyanins.

The interactions between polyphenols and fibre

Most non-extractable polyphenols with a high molecular weight are usually attached with covalent bounds to dietary fibres (Edwards *et al.*, 2017). Ortega, Macià, Romero, Reguant, and Motilva (2011) revealed that the soluble dietary fibres enhanced the stability of phenolic

compounds in an *in vitro* digestion model. Since dietary fibres act as an entrapping matrix for polyphenols, and restrict the diffusion of the enzymes to their substrates in the stomach and small intestine, leading to the polyphenols to reach the large intestine.

2.5 Possible anti-diabetic mechanisms of blueberries and blackcurrants

2.5.1 Inhibiting carbohydrate digestion and glucose absorption in the intestine

The manipulation of carbohydrate digestion and glucose absorption are key targets for the treatment and management of T2DM. Key enzymes responsible for digestion of dietary carbohydrates to glucose includes α -amylase and α -glucosidase. Salivary and pancreatic α -amylases catalyses α -1, 4-glucosidic linkages breakage releasing maltose and other oligosaccharides, then the α -glucosidase, which is a membrane border enzyme, continues to catalyse the oligosaccharides into absorbable monosaccharides in the small intestine (Assefa *et al.*, 2019; Proença *et al.*, 2019). Thus, inhibiting α -amylase and α -glucosidase may regulate intestinal glucose digestion and absorption as well as postprandial blood glucose levels (Malunga, Eck, & Beta, 2016). Acarbose and voglibose are known anti-diabetic drugs that inhibit α -amylase and α -glucosidase (Dabhi, Bhatt, & Shah, 2013). However, there are certain side effects of taking these drugs such as liver and renal lesions, and adverse gastrointestinal reaction. Berries seem to exert similar benefits without these side effects. Skrovankova *et al.* (2015) proposed that blueberry inhibited α -glucosidase activity competitively with the substrates to bind with the active site of α -glucosidase.

Glucose is a hydrophilic molecule that cannot cross biological membranes. Hence, intestinal absorption of glucose is mediated by two transport systems: active transport via the sodium-dependent glucose transporter (SGLT1), which can be found in the small intestine brush border and facilitated diffusion via the glucose transporter (GLUT2), and which is a high capacity facilitative transporter independent of sodium in the basolateral membrane (Chen,

Tuo, & Dong, 2016; Navale & Paranjape, 2016).

2.5.2 Protecting pancreatic β -cell from glucotoxicity

The production of insulin is a cascade starting from the uptake of glucose by the GLUT2 transporters, followed by a cycle of enzymatic reactions (Röder, Wu, Liu, & Han, 2016). Meanwhile, the inactivation of ATP-sensitive potassium channels on the cell membrane induces the calcium channel to open and Ca^{2+} flows into the cell. Finally, the increase of Ca^{2+} concentration stimulates insulin release. In insulin resistance, long-term hyperglycaemia leads to the dysfunction of insulin action, β -cells release more insulin in order to compensate for this defect and then decrease the expression of genes involved in insulin production. Thus, the β -cell mass is reduced induced by apoptosis. Therefore, the insulin deficiency in pancreas is due to both the cell damage and the defects in the synthesis of insulin (Chen, Cohrs, Stertmann, Bozsak, & Speier, 2017; Shyr, Wang, York, Nichols, & Remedi, 2019).

Currently, the most common approach of determining this protection of berries is measuring insulin secretion and insulin content in cultured pancreatic cell lines such as human-derived Caco-2 cells, rat insulinoma iNS-1E β -cells and a clone of iNS-1 cell line (Yang & Chan, 2018). In addition, these cells can be cultured without losing their response to glucose for a long time. One study (Johnson & de Mejia, 2016) demonstrated that anthocyanins from fermented blueberry beverages increased insulin secretion in iNS-1E cells, thus increased the absorption in Caco-2 cells. The potential mechanisms involved in modulating the enzyme dipeptidyl peptidase-IV (DPP-IV) and its substrate glucagon-like peptide-1 (GLP-1), and upregulating the mRNA expression of insulin receptor associated genes such as insulin-like growth factor 1 receptor (IGF1R) and proteins in pancreatic β -cells, including insulin-like growth factor 2 (IGF-II), insulin-like growth factor binding proteins (IGFBP-2 and -3), and vascular endothelial growth factor. Blueberry extracts have also shown beneficial effects on protecting glucolipotoxicity induced INS832/13 β -cells over metformin via increasing the cell viability,

ameliorating cell secretion dysfunction, and reducing intracellular triglyceride levels (Liu *et al.*, 2015).

Apart from affecting insulin secretion, another potential mechanism of berries on the β -cells has been proposed. Research from Liu *et al.* (2019) measured insulin secretion from growth arrested (tetracycline-treated) β -cells to distinguish the insulin secretion effect from the cell proliferative effect. The glucose stimulated insulin secretion with blueberry extracts enhanced slightly. By contrast, the cell proliferation rate increased significantly with blueberry extracts compared to the control group. The results suggested that ameliorating the rate of cell proliferation could be a potential target therapy of T2DM.

2.5.3 Improving glucose uptake

Several researchers have demonstrated that polyphenols may enhance peripheral glucose uptake in both insulin sensitive and non-insulin sensitive tissues (Bahadoran, Mirmiran, & Azizi, 2013; Kim, Keogh, & Clifton, 2016; Manzano *et al.*, 2016). The insulin mediated glucose uptake takes place in insulin sensitive tissues (skeletal muscle, adipose tissue and liver) (Honka *et al.*, 2018). The liver plays a vital role in maintaining glucose homeostasis in close cooperation with peripheral tissues. The liver stores glucose as glycogen via glycogenesis. In a fasted state, the liver produces glucose by glycogenolysis or gluconeogenesis (Han, Kang, Kim, Choi, & Koo, 2016). The key enzymes involved in the regulation of glycogenesis are glucokinase and glycogen synthase. Pyruvate carboxylase, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase are the major enzymes responsible for the modulation of gluconeogenesis (Goswami, Datta, Biswas, & Saha, 2004). The activation of protein kinase B (PKB) by insulin contributes to the control of hepatic glucose metabolism. Increased hepatic gluconeogenesis caused by insulin resistance is considered one of the earliest pathological changes in T2DM subjects (Hatting, Tavares, Sharabi, Rines, & Puigserver, 2018).

In obesity induced T2DM subjects, increases in free fatty acids (FFAs) may affect the IRS-1/phosphatidylinositol (PI)-3 kinase (PI3K)/PKB pathway, leading to a reduction of glucose uptake and phosphorylation in the muscle, simultaneously, decreasing the glycogen synthase activity (Huang, Liu, Guo, & Su, 2018). One potential mechanism responsible for this alteration is diacylglycerol (DAG)/protein kinase C (PKC) pathway. Increased FFAs have been shown to induce increased DAG levels, which in turn activate PKC activity. DAG may play a vital role in the activation of PKC, thereby leading to the progression of hepatic insulin resistance and hyperglycaemia. The accumulation of lipids in the liver and induced fatty acid oxidation may increase ROS production, which in turn leads to liver dysfunction (Ormazabal *et al.*, 2018).

Bilberry (European blueberry) anthocyanin has been reported to ameliorate hyperglycaemia and insulin sensitivity by activating the adenosine monophosphate-activated protein kinase (AMPK) in T2DM mice at skeletal muscle, liver, and white adipose (Takikawa, Inoue, Horio, & Tsuda, 2010). The activation of AMPK causes upregulation of GLUT4 in the skeletal muscle and white adipose tissue while inhibiting glucose production in the liver. AMPK activation in the liver also results in a significant reduction in liver and serum lipid content via the phosphorylation of acetyl-CoA carboxylase, upregulation of peroxisome proliferator-activated receptor alpha, acyl-CoA oxidase, and carnitine palmitoyltransferase-1A gene expressions. Russo, Picconi, Malandrucchio, and Frontoni (2019) revealed that blueberry extract (10 g of anthocyanins/kg diet) enhanced GLUT4 expression in skeletal muscle and white adipose tissue of T2DM mice. Additionally, GLUT4 expression was increased by activation of AMPK phosphorylation.

2.5.4 Antioxidant properties

The excessive production of reactive oxygen species (ROS) leads to oxidative stress, playing a vital role in the induction and progression of insulin resistance and diabetes (Oguntibeju, 2019). In hyperglycaemia subjects, high concentrations of glucose enter the mitochondria of

β -cell and are oxidatively phosphorylated (Gerber & Rutter, 2017). The amount of ROS, which are generated from mitochondria, can lead to β -cell dysfunction. The free radical scavenging capacity of anthocyanins depends on the number, and position, of methyl and hydroxyl groups in the skeleton, the B-ring of delphinidin containing 3 hydroxylation creates a strong antioxidant capacity. Oxygen radical absorbance capacity and ferric reducing antioxidant capacity assays indicated that berries have a high resistance to ROS (Tangvarasittichai, 2015). Oguntibeju (2019) found that the ROS level reduced after administration of blueberry extracts in acrylamide-stimulated rats. In T2DM subjects, low glucose levels in the muscle and adipose tissue may cause extracellular hyperglycaemia, leading to tissue damage and T2DM complications.

Hyperglycaemia can lead to diabetic complications by stimulating the generation of free radicals via different pathways and mechanisms such as oxidative phosphorylation, glucose autooxidation, NAD(P)H oxidase, lipoxygenase, cytochrome P450 monooxygenases, and nitric oxide synthase (Di Meo, Reed, Venditti, & Victor, 2016). For example, phenolic compounds can modulate the activity of kinase and activated the transcription factor such as Nrf-2, NF- κ B, then activating the activities of PRR dimerisation and PPAR- γ . Dysregulation of insulin secretion is influenced by protein tyrosine phosphatase-1B (PTP-1B), a negative regulator of the insulin signalling pathway that has been implicated in the progression of T2DM (Lee, 2017). The inhibition of aldose reductase is another relevant therapeutic approach for the management of T2DM. Hyperglycaemia may influence high levels of glucose into the polyol pathway, thereby leading to the accumulation of sorbitol. Accumulation of sorbitol is responsible for the development of T2DM complications (Yan, 2018). Some natural bioactive compounds from chinese herbs extracts have been demonstrated to be potential inhibitors of aldose reductase. Particularly, hydroxylation, glycosylation, and hydrogenation of the C2 = C3

double bond in the flavonoid structure involve in their inhibition on aldose reductase activity (Patil, Meshram, Barage, & Gacche, 2019).

2.5.5 Anti-Inflammatory properties

Evidence has shown that inflammation initiated from adipose tissue is one of the key factors for the development of insulin resistance and T2DM (Burhans, Hagman, Kuzma, Schmidt, & Kratz, 2018; Zatterale *et al.*, 2020). In T2DM subjects, increased number of macrophages infiltrates into adipose tissues and secretes chemokines released from adipocyte such as tumour necrosis factor-alpha (TNF- α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1).

Xie *et al.* (2011) reported that lipopolysaccharide induced mice fed with 10% blueberries reduced the expression of protein and mRNA of TNF- α and IL-6 in blood serum compared to the control group. A recent trial (Khoo *et al.*, 2017) showed that the level of plasma IL-1 β was significantly lower, than the placebo, in hypercholesterolemic when subjects consumed a purified anthocyanin mixture (320 mg/day) for 24 weeks. Roth *et al.* (2016) demonstrated that with the treatment of 10 mg/ml anthocyanin-containing bilberry extract (25% anthocyanin content) in IFN- γ -induced (100 ng/ml) human monocytic THP-1 cells, the expressions of MCP-1, IL-6, TNF- α , and intercellular adhesion molecule 1 (ICAM-1) were all significantly reduced. Azzini, Giacometti, and Russo (2017) also reported that supplementation with 8% (w/w) wild blueberry powder significantly increased blood adiponectin levels and reduced the expression of inflammatory markers in white adipose tissues as well as improved dyslipidaemia.

2.5.6 'Oxidative-Inflammatory Cascade'

'Oxidative-Inflammatory Cascade (OIC)' is series of events induced by the interaction of inflammation and oxidative stress (Mehta, Rayalam, & Wang, 2018). Insulin resistance can be considered as OIC events. Chronic hyperglycemia triggers mediators of meta-inflammation

and oxidative stress, which can activate stress-sensitive signalling pathways such as NF- κ B, JNK, PKC, p38 MAPK, and IKK β pathways. Activation of these pathways leads to insulin resistance and β -cell dysfunction, which will in return cause the increased glucose levels, leading to a vicious cycle (Brereton, Rohm, & Ashcroft, 2016). Thus, OIC aggravates the progression of T2DM.

Anthocyanins from blueberry decrease cellular ROS in lipopolysaccharide treated macrophages (Lee, Brownmiller, Lee, & Kang, 2020). Blueberry anthocyanins also reduce the gene expression of IL-1 β and TNF in a dose-dependent manner. The possible mechanism being that lipopolysaccharide stimulated the inhibition of NF- κ B p65 translocation to the nucleus.

One study (Xu *et al.*, 2016) showed that blueberry extracts suppressed inflammatory mRNA biomarkers such as COX-2, iNOS, and IL-1 β . In addition, malvidin-3-glucoside, epicatechin and chlorogenic acid from blueberries were most likely associated with the regulation of acute inflammatory response in murine RAW 264.7 macrophages. By contrast, the inflammatory response and antioxidant activity did not change in diet-induced obese mice fed with frozen blueberry (5%) for 12 weeks (Nemes *et al.*, 2019). In terms of clinical studies, regular consumption of 250 mL of a blueberry drink for 6 weeks did not affect inflammatory biomarkers or enzymatic oxidant activity (Miller, Feucht, & Schmid, 2019).

2.6 Evidence from clinical studies

Several randomized, parallel-design clinical studies (Basu *et al.*, 2010; Curtis *et al.*, 2019; Johnson *et al.*, 2015) have reported that consumption of blueberry and blackcurrant may positively affect early biomarkers of cardiovascular disease and diabetes. Törrönen, Hellström, Mattila, and Kilpi (2017) conducted a clinical trial of healthy subjects, showing that sweetened blackcurrant juice improved postprandial glycaemic control. In a clinical trial of 24 weeks involving 58 diabetic patients, the subjects in the anthocyanin group consumed two

anthocyanin capsules (160 mg anthocyanins) twice daily purified from bilberry and blackcurrant (Li, Zhang, Liu, Sun, & Xia, 2015). The results showed that the anthocyanin group had a significantly lower fasting plasma glucose and insulin resistance index compared to the control group. Stote *et al.* (2020) reported a daily consumption of 22 g of freeze-dried blueberries for 8 weeks had beneficial effects on cardiometabolic health parameters, including the fasting plasma glucose level, serum insulin, total cholesterol, and body weight in men with T2DM. By comparison, a clinical trial conducted on 44 adults (Stull *et al.*, 2015) showed that the daily consumption of blueberry did not improve the blood pressure or blood glucose. Hence, the effects of consumption of blueberry and blackcurrant on glycaemic control are still needed to be further investigated.

2.7 Conclusion

T2DM is increasing in prevalence worldwide. Although the specific mechanisms of T2DM still need to be investigated, there is a considerable evidence that inflammation and oxidative stress play a major role in the progression of the disease. Blueberries and blackcurrants have drawn much attention for their potential anti-diabetic activities. Animal studies have shown that intake of blueberries and blackcurrants may maintain insulin sensitivity.

Based on the review above, it is clear that there needs to be further work in order to understand the interaction between polyphenols and other components as clearly as possible, and to explain the specific polyphenol positive effects through investigating their interactions.

Chapter 3

Materials and methods

3.1.1 The moisture content of raw materials

Oat bran (Sun Valley, Christchurch, New Zealand) was obtained from the local supermarket (New World, Christchurch, New Zealand). Blueberry powder and blackcurrant powder were purchased online (Viberi, Timaru, New Zealand). The moisture of raw materials was determined by oven-drying method (Ahn, Kil, Kong, & Kim, 2014). Samples were weighed using an analytical balance (ARC120; OHAUS Corp., Parsippany, NJ, USA) into a pre-weighed dish. The dish was placed in an oven at 105 °C overnight, and then the dish was placed in the desiccator for 1 h to allow it to cool to room temperature before reweighing. The moisture content of samples was calculated by the equation (3-1) below:

$$\text{Moisture content (\%)} = \frac{\text{Loss of weight}}{\text{Sample weight}} \times 100\% \quad (3-1)$$

3.2 Preparation of the oat bran paste

Oat bran enriched with 0%, 10%, 15%, and 25% (w/w) blueberry powder or blackcurrant powder was used to develop the pastes. The equations (3-2) and (3-3) were used to determine the weight of water and raw materials (Balet, Guelpa, Fox, & Manley, 2019). **Table 3-1** shows the weight of water and raw materials for preparing the pastes. The samples were corrected for the moisture basis. After preparing the food matrix, the pastes were made using a Rapid Viscosity Analyser (RVA-Super 4, Perten instruments, Sydney Australia). The slurry was heated from 50 °C to 95 °C at a rate of 6 °C/min, held at 95 °C for 5 min, then cooled at a rate of 6 °C/min to 50 °C, and finally held at 50 °C for 2 min. The spindle speed was kept at 160 rpm,

except for the first 10 s where it was increased to 960 rpm to disperse the mixture. All the pastes were stored at 4 °C overnight.

$$S = \frac{86 \times 6.0}{100 - M} \quad (3-2)$$

$$W = 25 + (6.0 - S) \quad (3-3)$$

Where S = corrected sample weight (g), W = corrected water weight (g) and M = actual

Table 3-1 The weight (g) of water and raw materials for preparing the pastes

Group	Weight of water	Weight of OB	Weight of BB powder	Weight of BC powder
OBP	25.472	5.280	-	-
ABB₁₀	25.720	5.280	0.536	-
ABB₁₅	24.928	5.280	0.805	-
ABB₂₅	24.400	5.280	1.342	-
ABC₁₀	25.720	5.280	-	0.536
ABC₂₅	24.928	5.280	-	0.804
ABC₂₅	24.400	5.280	-	1.339

OBP = pure oat bran paste; OB = oat bran; BB = blueberry; BC = blackcurrant; ABB₁₀, ABB₁₅ and ABB₂₅ = oat bran paste enriched with 10%, 15% and 25% blueberry powder, respectively; ABC₁₀, ABC₁₅ and ABC₂₅ = oat bran paste enriched with 10%, 15% and 25% of blackcurrant powder, respectively. Samples were corrected for the moisture basis.

moisture content of sample (in %).

3.3 Colour measurement of pastes

The colour of the pastes was measured using a colorimeter CR-210 (Minolta, Tokyo, Japan).

The results were expressed using L^* , a^* and b^* values. Change in colour, ΔE , as a parameter

(the distance between two colours: oat bran paste (OBP) and blueberry or blackcurrant enriched pastes), was calculated from the equation (3-4) below (Bajaj, Urooj, & Prabhasankar, 2006):

$$\Delta E = \sqrt{(L_{Sample}^* - L_{OBP}^*)^2 + (a_{Sample}^* - a_{OBP}^*)^2 + (b_{Sample}^* - b_{OBP}^*)^2} \quad (3-4)$$

3.4 Proximal analysis of lyophilised samples

3.4.1 The moisture content of lyophilised samples

All the pastes were freeze dried at -30 to -40 °C using pilot scale lyophilization system (Millrock Technology, Inc., Kingston, NY, USA) for 72 h, and then the lyophilised samples were collected and stored at 4 °C. The moisture of lyophilised samples was determined by oven-drying method (Ahn *et al.*, 2014). Samples were weighed using an analytical balance (ARC120; OHAUS Corp., Parsippany, NJ, USA) into a pre-weighed dish. The dish was placed in an oven at 105 °C overnight, and then the dish was placed in the desiccator for 1 h to allow it to cool to room temperature before reweighing. The moisture content of samples was calculated by the equation (3-5) below:

$$\text{Moisture content (\%)} = \frac{\text{Loss of weight}}{\text{Sample weight}} \times 100\% \quad (3-5)$$

3.4.2 Ash content of lyophilised samples

The ash content was determined by using carbonisation at 550 °C (Mufla, INDEF) (Ismail, 2017). Each of samples was weighed before and after ashing to determine the concentration of ash present. The ash content was expressed on a dry basis :

$$\text{Ash content (\%, dry basis)} = \frac{M_{ASH}}{M_{DRY}} \times 100\% \quad (3-6)$$

Where M_{ASH} refers to the weight of the ashed sample, and M_{DRY} is the original weight of the dried samples.

3.4.3 Fat content of lyophilised samples

The crude fat content was determined using the Soxhlet extraction method (Carpenter, 2010). Each sample (2 g) was weighed into separate thimbles (recording the weights, sW) to conduct the extraction. The empty fat collector cup was weighed and recorded as eW . Petroleum ether was added to the glass tubes and the thimbles were suspended in the glass tube with a holder. The sample was extracted with petroleum spirit to dissolve fats, oils, pigments and other fat-soluble substances, and then the petroleum spirit was evaporated from the fat solution. After 1 h, the glass tubes were placed in a hot air oven at 105 °C. After 20 min, samples were cooled at 25 °C, and then reweighed the fat collector cups with sample extracts (fW). The fat content of each sample was calculated according to the following equation:

$$\text{Crude fat content (\%, dry basis)} = \frac{fW - eW}{sW} \times 100\% \quad (3-7)$$

3.4.4 Protein content of lyophilised samples

The protein content was determined using the Kjeldahl procedure by using the conversion factors of 5.83 for oat bran and all the pastes (Mariotti, Tomé, & Mirand, 2008), and 6.25 for blueberry and blackcurrant powders (Seo, Seo, & Yang, 2016).

3.4.5 Total starch content of lyophilised samples

The total starch content was determined using a starch analysis kit (Megazyme International Ireland Ltd, Wicklow, Ireland) and followed by the instruction of the manufactory. Each test sample (100 mg) was conducted in duplicate (one as a sample blank) and was mixed well with 10 mL of sodium acetate buffer (100 mmol/L, pH = 5.0) in a test tube. Thermostable α -amylase (0.1 mL) was added into the sample tube, while 0.1 mL of sodium acetate buffer (100 mmol/L,

pH = 5.0) was added into the blank tube. The mixture was incubated in a boiling water bath immediately for 15 min (at 0 min, 2 min, 5 min and 10 min, and 15 min, the tube contents were vortexed for 5 s). Afterwards, the tubes were placed in a water bath at 50 °C for 5 min, and then 0.1 mL of amyloglucosidase was added into the sample tube, while 0.1 mL of sodium acetate buffer (100 mmol/L, pH = 5.0) was added into the blank tube. The mixture was incubated at 50 °C for 30 min. After the tubes were cooled to the room temperature, transferred 2 mL of each solution to the microfuge tube and centrifuged these tubes at 5,000 g for 5 min. Supernatant (1 mL) was transferred into a tube containing 4 mL of acetate buffer (100 mmol/L, pH = 5.0) and the contents were mixed. the mixture (0.1 mL) was transferred to a new test tube, and then 3 mL of glucose oxidase/ peroxidase (GOPOD) reagent was added. The solution was incubated at 50 °C for 20 min. The absorbance was measured at 510 nm against the reagent blank. The starch content was calculated using the equation below:

$$\text{Starch content} \left(\%, \frac{W}{w}, \text{dry basis} \right) = \frac{\Delta A \times F \times D \times 0.9 \times EV \times 100}{W \times 100 - \text{moisture content} \left(\%, \frac{W}{w} \right)} \quad (3-8)$$

Where ΔA is the absorbance of sample solution read against the reagent blank. F is the factor to convert absorbance values to μg glucose. EV is the sample extraction volume. D is the further dilution of the sample solution. W is the sample weight in mg.

3.4.6 Dietary fibre content of lyophilised samples

The total dietary fibre (TDF) content was determined in duplicate using a total dietary fibre assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland). Soluble (SDF) and insoluble fibre (IDF) were determined and summed to determined TDF. Each sample was completely dispersed into 40 mL of MES-TRIS buffer (pH = 8.2) in a 400 mL beaker on the multi-stir. Once 50 μL of heat-stable α -amylase solution was added into the mixture, the beaker was covered an aluminium foil square, and then put in a shaking water bath at 98-100 °C for 30 min. The

beaker was cooled to 60 °C and the foil cover was removed. Afterwards, 100 µL of protease solution was added into the mixture and incubated in a shaking water bath at 60 °C for 30 min. HCl (0.561 N) was added and the pH of the solution was adjusted to 4.1~4.8, and then 200 µL of protease solution was added into the mixture and incubated in a shaking water bath at 60 °C for 30 min.

Insoluble dietary fibre of lyophilised samples

The enzyme mixtures were filtered through crucible (wet and redistributed bed of celite in crucible using 5 mL of distilled water) into a filtration flask. The solution of each sample was transferred to a 600 mL tall-form beaker. The residue in crucible was washed twice with 10-mL of 95% ethanol and acetone. The crucible containing residue was dried overnight in an oven at 103 °C. The crucible was cooled in desiccator for 1 h, and then weight the crucible containing the dietary fibre residue. One residue was analysed for protein using Kjeldahl method. For ash analysis, the second residue was incinerated for 5 h in an oven at 525 °C. The crucible was cooled in desiccator, and then weighed to determine the ash content.

$$\text{Dietary fibre (\%)} = \frac{\frac{R1 + R2}{2} - P - A - B}{\frac{m1 + m2}{2}} \times 100\% \quad (3-9)$$

Where $R1$ is the residue weight 1 from $m1$; $R2$ is the residue weight 2 from $m2$; $m1$ is the sample weight 1; $m2$ is the sample weight 2; A is the ash weight from $R1$; P is the protein weight from $R2$; B is the blank weight.

Soluble dietary fibre of lyophilised samples

The filtrate, and water left in the filtration flask in 3.5.6.1, was weighed, and then 4-fold volumes of 95% ethanol pre-heated to 60 °C were added. The precipitate was allowed to form at the room temperature for 1 h, then it was filtered using the crucible (wet and redistributed using 15 mL of 78% ethanol). The residue in the crucible was washed successively with two 15 mL portions of 78% ethanol and acetone, and then the crucible was dried overnight in an oven

at 103 °C. The protein and ash content in the residue were determined as described in 3.5.6.1.

The SDF content was calculated according to the equation 3-9.

Total dietary fibre of lyophilised samples

The TDF content was determined by summing IDF (3.4.6.1) content and SDF content (3.4.6.2).

3.5 Extraction of lyophilised samples

Each lyophilised sample (2 g) was mixed with 20 mL of acidic methanol/water (50:50 v/v, pH 2). The mixture was sonicated for 15 min in an ultrasonic bath, and then centrifuged at 2,500 g for 10 min. The supernatant was recovered. Acetone/water (70:30, v/v, 20 mL) was added into the residue, and then shaking and centrifugation were repeated. The methanolic and acetonetic extracts were combined (Annegowda, Bhat, Min-Tze, Karim, & Mansor, 2012). The final extracts were stored at -80 °C for analysis.

3.6 Simulation of the *in vitro* digestion procedure

The *in vitro* digestion method was modified to include an oral digestion step, prior to the gastric and intestinal digestion steps as described by Sengul, Surek, and Nilufer-Erdil (2014). The entire procedure was performed in a 37 °C incubator with constant shaking table at 120 r/min. Each lyophilised sample (2.0 g) was added to 20 mL of distilled water, and then 500 µL of an α -amylase/ CaCl_2 solution (1.3 mg/mL α -amylase in 1 mmol/L CaCl_2 , pH = 7.0) was added. This mixture was incubated for 10 min. The pH value of the mixture was adjusted to 2.0 with 6 mol/L HCl. Pepsin was added at a concentration of 0.05 g/mL of sample, and the mixture was incubated for 1 h. After finishing the gastric digestion, 1 mL of aliquot from each sample was taken (time 0) and added to 4 mL of absolute ethanol to stop the further reaction. The pH of the digest was adjusted to 6.0 by the dropwise addition of 0.9 mol/L NaHCO_3 . After the pH adjustment, 0.1 mL of α -amylglucosidase (3000 U/mL) was added. The digestion time began

as soon as 5 mL of pancreatin-bile solution (3 g/mL pancreatin and 0.025 g/mL bile salts in 0.1 mol/L NaHCO₃, pH = 7.4) was added. At 20, 60 and 120 min, 1mL of aliquot from each sample was taken and treated with ethanol individually. All of the aliquots were centrifuged at 2,500 g for 20 min and the supernatants were collected. The supernatant (1 mL) from each digest was stored at -20 °C for subsequent analysis.

3.7 Glycaemic glucose equivalent (GGE) assay

A GGE assay was carried out to evaluate the amount of reducing sugar released from the lyophilised sample over a period of 120 min *in vitro* digestion using the 3,5-dinitrosalicylic acid (DNS) method (Monro, Mishra, & Venn, 2010). As described in 3.8, the aliquots collected at 0, 20, 60, and 120 min were reacted with the enzyme solution of 1% invertase and 1% amyloglucosidase individually (dissolved in 0.25 mL of 0.2 mol/L acetate buffer, pH = 5.2), and digested at 25 °C for 20 min. Prior to the determination, 3,5-Dinitrosalicylic acid (DNS) powder (10 g) was dissolved in 400 mL of 2 mol/L NaOH solution with vigorous stirring and heat (less than 70 °C) in the dark for 16 h. Afterwards, potassium sodium tartrate tetrahydrate (300 g) was completely dissolved in 500 mL of distilled water, and then this solution was added into the DNS solution. Once the mixture was completely dissolved and clear, the solution was transferred to a 1 L of volumetric flask to make the volume up to 1 L. The final DNS reagent was preserved in a tightly stoppered container for GGE analysis. The released reducing sugar was measured by adding 0.75 mL of DNS mixture (0.05 mg/mL D-glucose: 4 mol/L NaOH: DNS reagent = 1:1:5) and heated for 15 min at 95-100 °C. When the tubes were cooled, 4 mL of RO water was added into the mixture. The absorbance was recorded at 530 nm. The amount of reducing sugar released in mg glucose/g sample was calculated and plotted versus time, while the area under the curve (AUC) was calculated by dividing the graph into trapezoids.

3.8 Determination of major phenolic rings by high performance liquid chromatography (HPLC) in extracts

The analyses of phenolic compounds was performed on an Agilent HPLC with quaternary pump and photodiode array detector (DAD) and fluorescence detectors (FLD) (Varian Inc., Walnut Creek, CA, USA). The column temperature was kept at 20 °C. After injecting 10 µL of sample, separation was performed in an Ace[®] 5 C18 250 × 4.6 mm column (Advanced Chromatography Technologies, Aberdeen, Scotland). In order to detect and quantify the compounds, the chromatograms were recorded at 280, 320, and 360 nm in the DAD and the chromatogram corresponding to scan from 220 nm to 600 nm in the FLD (Yan, Zhang, Zhang, & Zheng, 2016). For quantitation of the anthocyanidins at 520 nm in 25% blueberry and 25% blackcurrant enriched pastes, the extracts were treated with deglycosylation. Briefly, extracts were dissolved in the concentrated HCl to make the final concentration of the mixture as 2 mol/L. Subsequently, the mixture was put in the boiling water bath for 1 h, and then centrifuged at 5,000 g for 15 min. The supernatant was collected for the HPLC analysis. The flow rate was 0.8 mL/min. **Table 3-2** shows the solvent gradient used for separation. Data were processed and compared with the pure standard compounds in the specific wavelength (Gomez-Gomez, Borges, Minatel, Luvizon, & Lima, 2017).

3.9 Total phenolic content (TPC)

The TPC of the undigested (from extraction step as described in 3.5) and digested extracts (from the *in vitro* digestion procedure as described in 3.6) was measured by Folin-Ciocalteu reagent as described by Kim and Lee (2008) with some modifications. Each extract (0.5 mL) was placed in tubes and 2.5 mL of 0.2 mol/L Folin-Ciocalteu reagent and 2.0 mL of 7.5% Na₂CO₃ were added to each tube. These tubes were mixed well and incubated in a water bath at 40

°C for 30 min. Once the mixture was cooled to room temperature, the absorbance was measured at 760 nm by a spectrophotometer. Gallic acid was used as a standard to determine TPC of the extract and digesta as mg gallic acid equivalent (GAE)/g sample.

3.10 Total flavonoids content (TFC)

The TFC of the undigested (from extraction step as described in 3.5) and digested extracts (from the *in vitro* digestion procedure as described in 3.6) were measured using aluminium chloride reagent method (Zhou, Lin, Abbasi, & Zheng, 2016). Each extract (250 µL) was mixed with 75 µL of sodium nitrite solute ion (5%, w/v), followed by 150 µL of aluminium chloride (10%, w/v), 500 µL of sodium hydroxide (1 mol/L) and finally 775 µL of distilled water. The mixture was shaken and incubated at room temperature for 30 min. The absorbance of the mixture was measured at 415 nm. Results were expressed as mg rutin equivalents (RE)/g sample.

Table 3-2 The solvent gradient used for separation

Time (min)	Solvent A ¹ (%)	Solvent B ² (%)	Solvent C ³ (%)
0	100	0	0
2	100	0	0
5	93.6	6.4	0
17	2.8	11.2	86
22	3.6	14.4	82
29.5	4.2	16.8	79
55	6.6	26.4	67
70	10	40	50
75	10	40	50
78	36	64	0
81	36	64	0
86	100	0	0
90	100	0	0

3.11 Identification of the individual anthocyanidin and anthocyanin content in extracts by pH differential method

The individual anthocyanidin and anthocyanin in extracts were determined by the pH differential method (Giusti & Wrolstad, 2001). Prior to the test, lyophilised samples were extracted separately with different solvent systems (**Table 3-3**). These extracts were further diluted with potassium chloride buffer (pH = 1.0) and sodium acetate buffer (pH = 4.5), respectively. These dilutions were allowed to balance for 15 min. The absorbance of the dilutions was measured at $\lambda_{\text{vis-max}}$ and 700 nm, respectively, using a UV-Vis spectrophotometer (UV1800, Shimadzu, Kyoto, Japan). The absorbance of the diluted sample was calculated as follows:

$$A = (A_{\text{vis-max}} - A_{700\text{nm}})_{\text{pH}1.0} - (A_{\text{vis-max}} - A_{700\text{nm}})_{\text{pH}4.5} \quad (3-10)$$

¹ Solvent A: NH₄H₂PO₄ (0.05M, pH = 2.6)

² Solvent B: 100% Acetonitrile

³ Solvent C: H₃PO₄ (0.2M, pH = 1.5)

$$\text{Anthocyanin pigment} \left(\frac{\text{mg}}{\text{g}} \right) = \frac{A \times MW \times DF \times V \times 20}{a \times l \times m} \quad (3-11)$$

Where A is the absorbance, MW is the molecular weight, DF is a dilution factor, V is the solvent volume (mL), a is the molar absorptivity, and l is the cell path length (1 cm). m is the sample weight.

3.12 Identification of the total monomeric anthocyanin content (TMAC) in extracts

The TMAC value in extracts before and after the *in vitro* digestion was determined according to the method from 3.11. The absorbance of the mixture was measured at 530 nm and 700 nm. The TMAC was calculated according to the equation (3-10) and (3-11). The TMAC value was expressed as mg cyanidin-3-glucoside equivalents (Cy-3GE)/g sample. The molecular weight of cyanidin-3-glucoside is 449.2 g/mol, and the molar absorptivity of cyanidin-3-glucoside is 26,900 L · mol⁻¹ · cm⁻¹.

3.13 Determination of antioxidant capacity

3.13.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The radical scavenging capacity of undigested (from extraction step as described in 3.5) and digested extracts (from the *in vitro* digestion procedure as described in 3.6) was determined by the DPPH assay as described by Floegel, Kim, Chung, Koo, and Chun (2011). Methanolic DPPH solution (0.1 mmol/L, 1mL) was freshly prepared, and then added into 0.5 mL of the extract or digesta and incubated for 30 min in the dark. The absorbance of the reaction mixture was measured at 517 nm. Trolox was used as standard and the DPPH radical scavenging capacity was expressed as μmol Trolox equivalent (TE)/g sample.

3.13.2 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) assay

The ABTS^{•+} cation radical solution was produced by reacting 9.5 mL of 7 mmol/L ABTS stock solution and 245 μ L of 100 mmol/L K₂S₂O₈ solution and incubating this solution in the dark at room temperature for 16 h before use (Floegel *et al.*, 2011). The ABTS^{•+} radical cation solution was diluted with phosphate buffer solution (PBS, pH = 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm. The diluted ABTS^{•+} radical cation solution (3 mL) was thoroughly mixed with 0.3 mL of extract or digesta. The mixture was kept in the dark for 6 min at room temperature. Absorbance values were measured at 734 nm. Trolox was used for standard. Results were expressed as μ mol Trolox equivalent (TE)/g sample.

3.13.3 FRAP assay

The reducing capacity of the antioxidant power activity of each extract was determined by using FRAP reagent solution (Sompong, Siebenhandl-Ehn, Linsberger-Martin, & Berghofer, 2011). The fresh FRAP reagent solution was prepared with 300 μ mol/L acetate buffer (pH = 3.6), 10 mmol/L TPTZ (dissolved in 40 mmol/L HCl) and 20 mmol/L FeCl₃ at a ratio of 10:1:1 (v/v/v). FRAP reagent solution (2.5 mL) was thoroughly mixed with 250 μ L extract or digesta. The mixture was incubated in the dark for 2 h at 37 °C and the absorbance was measured at 593 nm. FeSO₄ solution was used as standard. Results were expressed as μ mol Fe³⁺ equivalent (Fe³⁺ E)/g sample.

Table 3-3 Reported molar absorptivity of anthocyanidins

Anthocyanidins	Solvent system	$\lambda_{\text{vis-max}}$ (nm)	Molar absorptivity (ϵ)
Cyanidin	0.1% HCl in ethanol	510.5	24600
Delphinidin	0.1% HCl in ethanol	522.5	34700
Malvidin	0.1% HCl in ethanol	520	37200
Peonidin	0.1% HCl in ethanol	511	37200
Pelargonidin	0.025 mol/L potassium chloride buffer, pH = 1.0	505	18420
Anthocyanins	Solvent system	$\lambda_{\text{vis-max}}$ (nm)	Molar absorptivity (ϵ)
Cyanidin 3-glucoside	HCl in 60% ethanol (pH = 1)	510	26900
Cyanidin 3-galactoside	0.1 mol/L HCl: Methanol = 1:99 (v/v)	530	34300
Cyanidin 3-rutinoside	Methanol: HCl (0.1 mol/L = 85:15 (v/v)	530	31085
Cyanidin 3,5-diglucoside	0.1 mol/L HCl	520	30175
Delphinidin 3-glucoside	1% HCl in methanol	543	29000
Delphinidin 3,5-diglucoside	1% HCl in methanol	540	28900
Malvidin 3-glucoside	10% ethanol, pH = 1.5	520	28000
Malvidin 3-galactoside	10% ethanol, pH = 1.5	520	27800
Malvidin 3,5-diglucoside	0.1% HCl in ethanol	545	10300
Peonidin 3-glucoside	0.1% HCl in methanol	540	20700
Peonidin 3-galactoside	0.1 mol/L HCl: ethanol = 15:85 (v/v)	531	48340
Peonidin 3-arabinoside	0.1 mol/L HCl: ethanol = 15:85 (v/v)	532	46100
Petunidin 3-glucoside	0.1% HCl in methanol	546	12900
Pelargonidin 3-glucoside	1% HCl in H ₂ O	496	27300

3.14 Enzymes inhibition assay

3.14.1 Inhibition of α -amylase activity

The α -amylase inhibitory activity assay was carried out according to a previous study (Unuofin, Otunola, & Afolayan, 2018). Extracts were dissolved in 0.01 mol/L PBS, (with 6 mmol/L NaCl, pH = 6.9) to give varying concentrations from 0.5 mg/mL to 20 mg/mL). Acarbose at various concentrations (from 0.005 mg/mL to 0.1 mg/mL) was used as positive control. In the test tube, reaction mixture containing 1 mL of fresh human salivary amylase solution (0.8 U/mL) and 500 μ L of extract or PBS (the control) was incubated at 37 °C for 10 min. Then, 1 mL of 5 mg/mL gelatinised starch solution was added in each tube to initiate the reaction. After 0, 5, 10, and 15 min, 300 μ L of the mixture was removed to a tube containing 500 μ L of 1 mol/L HCl to stop the reaction. Subsequently, 1 mL of 1% iodine-potassium iodide indicator solution was added to each quenched solution. The initial enzymic reaction velocity (v) were determined in the absence or presence of different concentrations of extracts at the absorbance of 630 nm using a UV-Vis spectrophotometer and expressed as the slope of the linear region of absorbance against the reaction time (Δ absorbance value/min). The percentage inhibition I (% , I) was calculated by using the following formula:

$$\% I = \frac{v_0 - v}{v_0} \times 100\% \quad (3-12)$$

Where v and v_0 are the initial reaction velocity in the presence and absence of extracts, respectively.

3.14.2 Alpha-amylase inhibitory kinetics

The kinetics of α -amylase inhibition by extracts were determined by using a gradient of 1.25, 2.5, 5, and 10 mg/mL gelatinised starch solution as substrates (Kazeem, Adamson, &

Ogunwande, 2013). In brief, extracts were dissolved in 0.01 M PBS, (with 6 mM NaCl, pH = 6.9) to give varying concentrations from 0.5 mg/mL to 20 mg/mL). Acarbose at various concentrations (from 0.005 mg/mL to 0.1 mg/mL) was used as positive control. In the test tube, a reaction mixture containing 1 mL of fresh human salivary amylase solution (0.8 U/mL) and 500 μ L of extracts or PBS (the control) was incubated at 37 °C for 10 min. Then, 1 mL of 1.25, 2.5, 5, and 10 mg/mL gelatinised starch solution was added to each tube to initiate the reaction, respectively. After 20 min, 500 μ L of 1 mol/L HCl was added into the mixture to stop the reaction. Subsequently, 1 mL of 1% iodine-potassium iodide indicator solution was added to each quenched solution. The reaction velocity of v for each substrate concentration, at different concentrations of inhibitors were investigated at 37 °C using the same method as mentioned in 3.14.1, and according to the equation (3-13) (Δ absorbance value/min). A Dixon equation (Dixon, 1953) was applied to determine the kinetic constants of extracts, which can further determine the inhibition type. The equations for competitive (3-14) and mixed-type inhibition (3-14) are as follows:

$$v = \frac{v_{max}a}{k_m \left(1 + \frac{i}{k_{ic}}\right) + a} \quad (3-13)$$

$$v = \frac{v_{max}a}{k_m \left(1 + \frac{i}{k_{ic}}\right) + a \left(1 + \frac{i}{k_{iu}}\right)} \quad (3-14)$$

Where v is the reaction velocity of the reaction, v_{max} is the maximum rate of initial velocity reaction, i represents the inhibitor (extracts and acarbose) concentration, a is the concentration of starch, k_m is the Michaelis constant, k_{ic} is depicting the competitive inhibition constant, while k_{iu} is denoting the uncompetitive inhibition constant.

Taking the reciprocals of both sides of the Dixon equation, the Dixon plot can be described as the linear plot of $1/v$ against i . It can be concluded by using two or more concentrations of substrates that $k_{ic} = -i$ for the both above equations, representing that k_{ic} equals to the

absolute value of the intersection abscissa of the Dixon plots along with various substrates concentrations.

The value of k_{iu} can be achieved by plotting $\frac{a}{v}$ against i at various substrates values, forming a Hanes-Woolf plot. The Hanes-Woolf equation (Hanes, 1932) for the mixed-type inhibition is described as follows:

$$\frac{a}{v} = \frac{k_m \left(1 + \frac{i}{k_{ic}}\right) + a \left(1 + \frac{i}{k_{iu}}\right)}{v_{max}} \quad (3-15)$$

It can be deduced that $k_{iu} = -i$ for the Hanes-Woolf equation, depicting that k_{iu} is equivalent to the absolute value of the intersection abscissa of the Hanes-Woolf plots along with various substrates concentrations.

For determining the apparent maximum reaction velocity (V_{max}^{app}) and the apparent Michaelis constant (K_m^{app}) (Ivanauskas, Kaunietis, Laurinavičius, Razumienė, & Šimkus, 2008), a double-reciprocal version (Lineweaver-Burk plot) of the Michaelis-Menten equation (Johnson & Goody, 2011; Lineweaver & Burk, 1934) can be applied as follows:

$$\frac{1}{v} = \frac{1}{V_{max}^{app}} + \frac{K_m^{app}}{V_{max}^{app}} \frac{1}{a} \quad (3-16)$$

According to the equation 3-15, the plot of $\frac{1}{v}$ against $\frac{1}{a}$ at a constant value of i is linear, which can be calculated of K_m^{app} by the slope of the linear and V_{max}^{app} from the intercept.

3.14.3 Inhibition of α -glucosidase activity

The α -glucosidase inhibitory activity assay was adapted from a previous study reported by Ni, Pan, Hu, Gong, and Zhang (2019). Prior to the reaction, extracts were dissolved in 0.01 M PBS (pH = 6.8) to give varying concentrations, from 2.5 mg/mL to 100 mg/mL. The reaction mixture containing 500 μ L of 1.25 mM p-nitrophenyl- α -glucopyranoside (p-NPG), 500 μ L of extracts, 500 μ L of 2 mg/mL glutathione (reduced solution) and 500 μ L of α -glucosidase (0.4 U/mL,

dissolved in 0.01 mol/L PBS, pH = 6.8), was incubated at 37 °C. After 0, 5, 10, and 15 min, 200 μ L of the mixture was removed to a tube containing 50 μ L of 0.2 M Na_2CO_3 to stop the reaction. The initial enzymic reaction velocity was determined at the absorbance of 400 nm. The percentage inhibition I (% , I) was calculated by using formula (3-12). Acarbose at various concentrations (from 0.5 mg/mL to 5 mg/mL) was included as a positive control. The IC_{50} values (concentration required to inhibit 50% of the α -amylase and α -glucosidase activity) were determined from plots of percentage inhibition versus log inhibitor concentration and were calculated by non-linear regression analysis from the mean inhibitory values.

3.14.4 Alpha-glucosidase inhibitory kinetics

The kinetics of α -glucosidase inhibition by extracts were determined by using a gradient of 0.625, 1.25, and 2.5 mM p-NPG as substrates (Yousof Ali *et al.*, 2020). Extracts were dissolved in 0.01 M PBS (pH = 6.8) to give varying concentrations, from 2.5 mg/mL to 100 mg/mL. The reaction mixture containing 500 μ L of 0.625, 1.25, and 2.5 mM p-nitrophenyl- α -glucopyranoside (p-NPG), respectively, 500 μ L of extracts, 500 μ L of 2 mg/mL glutathione (reduced solution) and 500 μ L of α -glucosidase (0.4 U/mL, dissolved in 0.01 mol/L PBS, pH = 6.8), was incubated at 37 °C. After 20 min, 500 μ L of 0.2 mol/L Na_2CO_3 was added into the mixture to stop the reaction. The initial enzymic reaction velocity (v) for each substrate concentration, at different concentrations of inhibitors were investigated at 37 °C using the same method as mentioned in 3.14.3, according to the equation (3-13) as mentioned above. A Dixon equation (Dixon, 1953) was applied to determine the kinetic constants of extracts, which can further determine the inhibition type. The equations for competitive and mixed-type inhibition were outlined in the formula (3-14) and (3-15). For determining the apparent maximum reaction velocity (V_{max}^{app}) and the apparent Michaelis constant (K_m^{app}), a

Lineweaver-Burk plot of the Michaelis-Menten equation was applied as described in the equation (3-16).

3.15 Molecular docking study on α -amylase and α -glucosidase

Molecular docking was performed to investigate the interaction of α -amylase and α -glucosidase with the anthocyanidins, respectively. The three dimensional structure of delphinidin 3-glucoside (del-3-glu, CAS: 6906-38-3), cyanidin 3-glucoside (cya-3-glu, CAS: 7084-24-4), malvidin 3-glucoside (mal-3-glu, CAS: 7228-78-6) and cyanidin 3-rutinoside (cya-3-rut, CAS: 28338-59-2) were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov>), and further minimised by AutoDockTools software V1.5.6 (San Diego, CA, USA). The crystal structure of α -amylase (PDB ID: 1SMD) (Ramasubbu, Paloth, Luo, Brayer, & Levine, 1996) and α -glucosidase enzyme (PDB ID: 3WY1) (Shen *et al.*, 2015) were obtained from the Protein Data Bank (<https://www.rcsb.org>). The PyMOL software V2.3.0 (Schrödinger, Inc., NY, USA) was applied to remove the original ligand and water molecules of the target enzymes, then the protein was imported into AutoDockTools for hydrogenation, charge calculation and distribution as well as atom type specification. AutoDock Vina was used to evaluate the binding affinity of each anthocyanidin to proteins.

3.16 The anticancer activities of extracts towards HepG2 cell line

3.16.1 Cell viability

The human hepato-carcinoma cell line, HepG2, was obtained from American Type Culture Collection (Manassas, VA, USA). HepG2 was grown in DMEM culture medium supplemented with 10% heat inactivated FBS, penicillin (100 U/mL), and streptomycin sulphate (100 μ g/mL) at 37 °C in a humidified atmosphere of 5% CO₂. HepG2 cells were treated with undigested (from extraction step as described in 3.6) and digested extracts (from the *in vitro* digestion

procedure as described in 3.8) individually and plated at a density of 5.0×10^3 cells/well in 96-well culture plates for 48 h. Cell viability was determined by using a Cell Counting Kit-8 (CCK-8) assay kit (Dalian Meilun Biotechnology Co., Ltd, Dalian, China). Absorbance was calculated for all samples at 450 nm (OD_{450}). The relative cell viability was presented after comparison with the untreated cells (control). Cell viability rates were calculated based on OD_{450} values. Cell viability rate (%) = $OD_{450}(\text{test})/OD_{450}(\text{control}) \times 100\%$.

3.16.2 Induction of cell apoptosis of HepG2 via flow cytometry assay

The cells were plated at a density of 3.0×10^5 cells/well in a 6-well plate, and incubated for 24 h at 37 °C. After treating with different concentrations of extracts for 24 h, the cells were washed twice with PBS. Cell apoptosis was analysed using an Annexin V-FITC/PI Apoptosis Detection Kit (V13241) (Thermo, Waltham, MA, USA). Briefly, Annexin V-FITC (5 μ L) and of 100 μ g/mL propodium iodidi (PI) (1 μ L) were added followed by the incubation of the cells in the dark for 15 min. The apoptotic rate was examined using a FACS flow cytometer. After staining, all samples were immediately measured on a CytExpert flow cytometer (CytoFLEX S, Beckman Coulter, CA, USA). CytExpert Software (CytoFLEX S) was used to analyse the data (Nadhe *et al.*, 2020).

3.16.3 Hoeschst staining

HepG2 cells were plated at a density of 3.0×10^3 cells/well in a 96-well plate and incubated for 24 h at 37 °C. According to the results of cell viability, and to make sure the rate of cell viability is more than 90%, the final extract concentration of 450 μ g/mL was selected for subsequent cell line studies. After treating with different extracts for 24 h (completed DMEM medium for the control group), the supernatant was removed and treated by adding 5 μ g/mL Hoechst for 15min. The samples were then washed twice with PBS and photographed using a fluorescence microscope (magnification, $\times 100$) (Qin *et al.*, 2017).

3.16.4 Inhibition of HepG2 cell invasion

Matrigel invasion assay was performed using a 24-well Transwell chamber (Costar, Cambridge, MA, USA), which was pre-coated with Matrigel (BD Bioscience, San Jose, CA, USA) for 4 h at 37 °C. HepG2 cells were suspended in 200 µL of medium without serum and were transferred to the upper Transwell chambers. DMEM (500 µL) with 10% FBS was added to the lower chamber and the cells were incubated for 48 h. The non-invaded cells on the upper membrane surface were removed using a cotton swab. The cells on the lower surface of the membrane were fixed in 4% triformol for 15 min and stained with 0.1% crystal violet staining solution for 15 min. The cells in at least five random microscopic fields (magnification, ×200) were counted and photographed (Pijuan *et al.*, 2019).

3.16.5 HepG2 cell cycle analysis

The cells were treated with different extracts for 24 h, and then washed twice with PBS and subsequently fixed with 70% ethanol at -20 °C in the dark for 24 h. The fixed cells were washed twice with PBS, and then stained with propidium iodide (PI, 50 µg/mL) and Rnase (2.5 µg/mL) for 1 h in the dark. All samples were immediately measured on a CytExpert flow cytometer (Kanipandian, Li, & Kannan, 2019).

3.16.6 5-Ethynyl-2'-deoxyuridine (EdU) staining

HepG2 cells were plated at a density of 3.0×10^3 cells/well in a 96-well plate and incubated for 24 h at 37 °C. According to the results of cell viability, the concentration of 450 µg/mL was selected as the concentration of extracts for the following analysis. After treating with different extracts for 24 h (completed DMEM medium for the control group), the supernatant was removed. Cells were rinsed once with PBS, and then stained with EdU mixture using an EdU-555 Cell Proliferation Detection Kit (BeyoClick™, Shanghai, China) according to the manufacturer's instruction. Cells were counterstained with Hoechst and imaged by

fluorescence microscopy (magnification, $\times 100$) (Kntayya *et al.*, 2018).

3.16.7 Induction of intracellular ROS generation of HepG2 cell line

The intracellular changes in ROS generation were detected by staining the cells with 2,7 dichlorodihydrofluorescein-diacetate (DCFH-DA). HepG2 cells were treated for 24 h. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂, and washed twice with cold PBS, then cells were further incubated with 10 μ M DCFH-DA at 37 °C for 30 min. Subsequently, the cells were washed two times using PBS. Prior to ROS measurement, 100 μ L of PBS was added to each well. ROS generation was assessed by flow cytometry (Siddiqui *et al.*, 2019).

3.16.8 Western blotting assay

After the treatment for 24 h, the cells were harvested, collected as cell pellets, and lysed in RIPA cell lysis buffer on ice for 1 h. Protein concentrations were determined using a BCA Protein Assay Kit (Thermo, MA, USA) (Liu, Zhang, Li, Han, & Wang, 2020). Equal proteins from each treatment were separated on a 10% SDS denaturing polyacrylamide gel and electrophoretically (SDS-PAGE) transferred to PVDF membranes. After blocking with 5% non-fat milk, the membranes were incubated with primary antibodies (1:1000; Cell Signalling Technology) overnight at 4 °C. Specific primary antibodies against Bcl-2, Bax, Caspase-3, Nrf2, HO1 and GADPH were purchased from Beyotime (Shanghai, China). After washing thrice (10 min each) with TBS solution, the PVDF membranes were incubated with the corresponding secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h. The blots were washed thrice (10 min each) with TBS solution. Signals were detected by using an Enhanced Chemiluminescence (ECL) detection (Thermo, MA, USA) and Image J (Bethesda, MD, USA) software were used to quantify the blot density.

3.16.9 Real-time Quantitative PCR assay

Total RNA was extracted using the Trizol (Sigma-Aldrich, St. Louis, MO, USA) method. cDNA

was synthesised using the PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Tokyo, Japan). The expression of mRNA was measured by RT-PCR with SYBR Green PC Master Mix (Applied Biosystems, Inc., Waltham, MA, USA). Thermocycler conditions consisted of initial holds at 50 °C for 2 min and 95 °C for 5 min followed by a PCR program of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s for 40 cycles and a final hold at 72 °C for 5 s. Reactions were executed by an ABI PRISM® 7300 Sequence Detection System (Applied Biosystems, Inc.). Data for all samples was normalised to the control. The expression of mRNA was calculated using the relative quantification equation ($RQ = 2^{-\Delta\Delta C_t}$). The primer sets are shown in **Table 3-4** (Brown *et al.*, 2018).

Table 3-4 Primers utilised in RT-PCR

Gene	Forward	Reverse
Nrf2	5'-CCTCAACTATAGCGATGCTGAATCT-3'	5'-AGGAGTTGGGCATGAGTGAGTAG-3'
HO1	5'-GCAGAGAATGCTGAGTTCATG-3'	5'-CACATCTATGTGGCCCTGGAGGAGG-3'
GAPDH	5'-GCCTCAAGATCATCAGCAATGC-3'	5'-CCTTCCACGATAC CAAAGTTGTCAT-3'

3.17 Statistical analysis

The results are presented as the mean value \pm standard deviation. Unless stated elsewhere, experiments were performed in triplicate. One-way analysis of variance (ANOVA) was carried out and in addition, where applicable, Pearson's correlation and two-way analysis of variance were conducted using GraphPad Prism software version 8.0 (GraphPad Software, Inc., San Diego, CA, USA). Enzyme kinetic analysis was conducted by using SigmaPlot software version 12.5 (Systat software, Inc., San Jose, CA, USA).

Chapter 4

The bioactive ingredients from blueberry and blackcurrant powder alter the physicochemical characteristics and the *in vitro* glycaemic response of oat bran paste

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Abstract

Cereal foods supplemented with fruit ingredients have attracted the attention of consumers due to their bioactive compounds which confer health promoting effects. In this chapter, oat bran was mixed with 0%, 10%, 15% and 25% (w/w) of blueberry and blackcurrant powder to study their physicochemical and nutritional characteristics. HPLC and an *in vitro* digestion system were employed to observe the effects of bioactive compounds from blueberry and blackcurrant powders on the predicted glycaemic response of oat bran. Results showed that blueberry and blackcurrant powder increased the phenolic contents compound (in particular the anthocyanin content) of oat bran paste, whilst decreasing the extent of starch degradation, and the area under the curve of reducing sugar released during the *in vitro* digestion. Correlations between physicochemical characteristics and the nutritional properties were recorded. Results revealed that supplementation of blueberry or blackcurrant powders in oat bran paste could provide healthier food characteristics, and manipulate the hypoglycaemic property of the paste

Keywords: pasting; viscosity; phenolic compounds; glycaemic response; *in vitro* digestion

4.1 Introduction

Diets high in fat, together with the sedentary lifestyles common to western countries, are considered as major causes of obesity-related insulin resistance and impaired tolerance to glucose among environmental factors (Kopp, 2019). These metabolic syndromes are the main predisposing factors to type 2 diabetes (T2DM), which is one of the world's biggest health epidemics (Akash, Rehman, & Chen, 2013). Even though regular exercise has been recommended for preventing the chronic health problems linked to obesity and T2DM, a recent report by *The Lancet Global Health* estimated that a third of UK adults were not active enough (Guthold, Stevens, Riley, & Bull, 2018). With the growing number of people being diagnosed with these metabolic syndromes, it is crucial to find a new solution. Epidemiological studies, and randomised control trials, have shown that dietary modification (in particular whole fruit and grains intake) are protective against diabetes and obesity since the complex mixture of phytochemicals from these foods has additive and synergistic effects (Jurkiewicz-Przondziono, Lemm, Kwiatkowska-Pamuła, Ziółko, & Wójtowicz, 2017). Berries have been shown to impart relief from obesity and T2DM. The nutraceutical values of these powerhouses of nutrients warrant further investigation for their optimal exploitation (Nasri, Baradaran, Shirzad, & Rafieian-Kopaei, 2014).

Blueberry (*Vaccinium sp.*) and blackcurrant (*Ribes nigrum L.*) fruits contain functional phytochemicals (Patel, 2014) (Park *et al.*, 2015). They are rich in dietary fibres, minerals, vitamins, and antioxidants, and have health promoting effects. They contain a high content of polyphenols, anthocyanins, phenolic acids and flavanol, and these compounds have been credited in conferring many healthy properties (Hanhineva *et al.*, 2010). Lomiwes *et al.* (2019) revealed that consuming a specific type of blackcurrant could help 'sedentary' adults walk for longer without getting tired. Additionally, the consumption of powdered blackcurrant extract

for 14 weeks was shown to decrease the weight gain of high-fat-induced fed C57Bl/6 mice (Esposito *et al.*, 2015). Sun-Waterhouse (2011) also illustrated the potential of blueberry polyphenols to regulate glucose metabolism and gut microbiota.

The role of breakfast cereals, particularly those which are whole grain or high in cereal fibre, has been recognised for many years as important in maintaining a balanced healthy diet. Dietary guidelines promote the nutrient density of breakfast cereals as an essential source of key nutrients (Williams, 2014). Kosti, Panagiotakos, and Zampelas (2010) reported that people who consumed breakfast cereals regularly exhibited a lower body mass index and were less likely to be overweight compared with individuals did not consume breakfast cereals. Chang *et al.* (2013) also found a reduced obesity risk with oat-based breakfast cereals intake when compared with oat germ's intake. Oat bran is often consumed a part of porridge and forms part of well-balanced and nutritious diets. In addition to providing an important source of vitamins, fibre (especially β -glucan), and minerals, oat bran also contains potentially important sources of antioxidants and phytoestrogens (Ulmius *et al.*, 2011). Ulmius *et al.* (2011) also revealed that oat bran had positive effects on postprandial glucose levels, serum cholesterol and body mass index.

Berries have a positive image amongst consumers, and their inclusion in cereal products appears to be a promising strategy to influence diets (Sun-Waterhouse, 2011). Although there are many papers investigating the effects of purified ingredients on physical and nutritional properties of berry fruits and cereal foods, few have reported the actual use of whole ingredients in food products and taken into consideration the effect of the whole food matrix on their physicochemical characteristics and nutritional properties when cereal foods are supplemented with berry fruits.

Therefore, in this chapter, blueberry and blackcurrant powder were selected as phenolic-rich supplements for the cereal food, oat bran. The combination of HPLC, an *in vitro* digestion, and

the pH differential method were employed to observe the food matrix effects on the changes in their phenolic contents and hypoglycaemic properties of blueberry and blackcurrant enriched oat bran food models.

4.2 Materials and methods

4.2.1 Pasting properties

Pastes of oat bran with blueberry powder and blackcurrant powder, respectively, were made by using RVA system as outlined in 3.2.

4.2.2 Colour measurement of pastes

The colour profile of pastes was determined as described in 3.3.

4.2.3 Proximate analysis

Proximate analysis of the lyophilised samples was performed as outlined in 3.4.

4.2.4 Extraction of raw materials and pastes

The lyophilised raw materials and pastes were extracted by two solvent systems, including 50% acidic methanol and 70% acetone, as mentioned in 3.5.

4.2.5 Determination of phenolic rings by HPLC

The major phenolic rings in extracts of raw materials and pastes were characterised by HPLC., outlined in 3.8.

4.2.6 Glycaemic glucose equivalent assay (GGE)

A starch degradation (GGE) assay was performed as described in 3.7.

4.2.7 Determination of total monomeric anthocyanins contents (TMAC) in extracts

The TMAC value in extracts of raw materials and pastes before and after the *in vitro* digestion was determined according to the method from 3.12.

4.2.8 Statistical analysis

Statistical analysis was conducted as described in 3.17.

4.3 Results and discussion

4.3.1 Blueberry and blackcurrant powders improved the pasting properties of oat bran paste

The pasting properties of RVA pastes are summarised in **Table 4-1** and the viscosity curves are shown in **Fig. 4-1**. As illustrated in **Table 4-1**, the peak viscosity and breakdown values of pure oat bran paste were lower than that of blueberry and blackcurrant enriched pastes ($p < 0.05$). When blueberry or blackcurrant powder was added into oat bran, the peak viscosity values increased. The peak viscosity of oat bran pastes enriched with 25% blueberry and 25% blackcurrant powder were 10.19 % and 12.66 % higher ($p < 0.01$), and the breakdown value of 25% blueberry and 25% blackcurrant enriched oat bran paste were 43.80 % and 68.08 % higher ($p < 0.01$), when compared with the oat bran paste, respectively. Pasting properties have been shown to be influenced by the interactions of starch with other components, such as carbohydrates, lipids, proteins and pH (Alcázar-Alay & Meireles, 2015). In this study, all mixtures appeared to be fully gelatinised by the RVA with a well defined peak viscosity at 95 °C. Negative correlations were observed between peak viscosity and both protein and total starch content ($R = -0.828$; $p < 0.05$, $R = -0.79$; $p < 0.05$, respectively) (**Table 4-2**). Considering that the peak viscosity represents the highest swelling points for starch granules, the results indicated that the presence of protein inhibited the absorption of water from starch granules (Seung *et al.*, 2015). According to the significant negative correlation between final viscosity and fat content ($R = -0.880$; $p < 0.01$), it can be speculated that lipids retard the solubility of starch. With increasing levels of blueberry or blackcurrant, the final viscosity of blueberry and blackcurrant enriched oat bran paste increased, which is possibly due to the starch gelatinisation and the interactions with β -glucan in the pastes, resulting in an entanglement

of molecules during cooling. These results are in agreement with a previous study, which reported that a high viscosity might be due to a combination of the high β -glucan and starch content (Kaur, Sharma, Ji, Xu, & Agyei, 2020).

Correlations were also found between the ratio of soluble dietary fibre to total dietary fibre and breakdown value ($R = 0.958$; $p < 0.01$) as well as the ratio of soluble dietary fibre to total dietary fibre and peak viscosity ($R = 0.789$; $p < 0.05$) (**Table 4-2**), which illustrated that soluble dietary fibre may have a positive correlation with the peak viscosity and the breakdown value since more starch granules could imbibe water before they break. The short-term retrogradation of pastes can be assessed using the setback value. As shown in **Table 4-1**, the setback value of blueberry and blackcurrant enriched oat bran paste were lower than the oat bran paste ($p < 0.01$). The setback value of 10% blueberry and 25% blackcurrant enriched oat bran pastes were 7.42% and 14.28% lower than that of the oat bran paste, respectively ($p < 0.01$). These results indicated that supplementation of oat bran with blueberry or blackcurrant could make the structure of pastes more thermally stable.

4.3.2 Colour profile of pastes

The lightness value of L^* represents the darkest black at $L^* = 0$ and the brightest white at $L^* = 100$. a^* value indicates redness (positive value) or greenness (negative value), while b^* value indicates yellowness (positive value) or blueness (negative value) (Good, 2002). As shown in **Table 4-3**, the colour parameters of L^* , a^* and b^* differed significantly ($p < 0.01$) among all pastes. Incorporation of blueberry or blackcurrant powder significantly influenced ($p < 0.01$) the surface colour of pastes. Both blueberry and blackcurrant enriched pastes were darker in colour compared with oat bran paste (lower L^* value). Blueberry enriched paste seemed to be darker than blackcurrant enriched pastes (**Fig. 4-2**). The higher a^* value of blueberry and blackcurrant enriched pastes indicated a redness of the pastes. Blueberry enriched paste showed more blueness, while the blackcurrant enriched paste showed more yellowness

compared to oat bran paste according to the b^* value. These colour differences were attributed to the original pigments in the blueberry and blackcurrant powder. Finally, with an increased proportion of blueberry and blackcurrant powder added into paste, ΔE value of both blueberry and blackcurrant enriched pastes also showed an increase, which was the same behaviour observed for total monomeric anthocyanins content ($R = 0.889$; $p < 0.05$) (**Table 4-4**). This result also indicated that ΔE might be a good parameter to evaluate the amount of the phenolic compounds (Gomez-Gomez *et al.*, 2017)

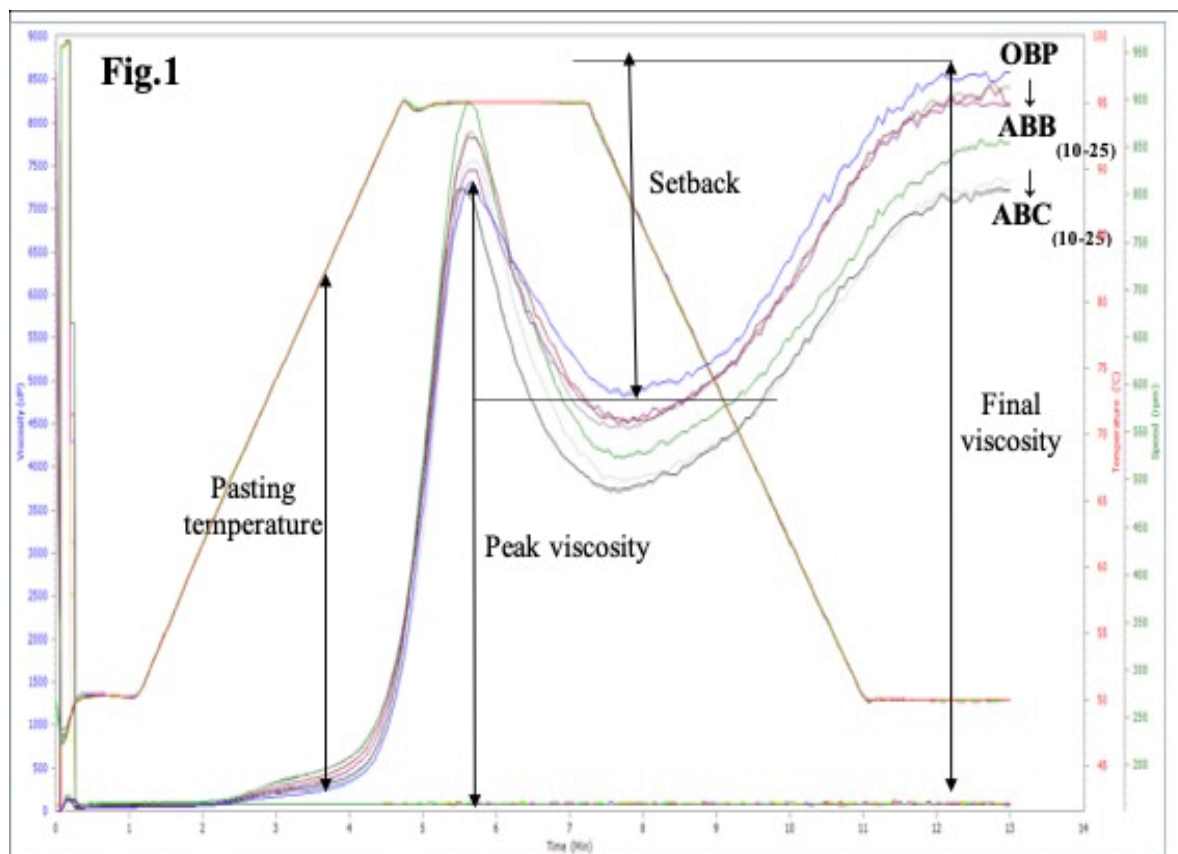


Figure 4.1 Curves of pasting properties determined by the RVA

Table 4-1 The pasting properties determined by the RVA

Group	Peak viscosity (cP)	Breakdown (cP)	Final viscosity (cP)	Setback (cP)	Pasting temperature (°C)
OBP	7180.00 ± 150.91 ^e	2401.67 ± 107.00 ^f	8836.67 ± 104.71 ^a	4057.00 ± 122.43 ^a	82.57 ± 0.46 ^a
ABB₁₀	7416.67 ± 42.15 ^d	2989.00 ± 115.29 ^e	8201.33 ± 79.59 ^d	3756.00 ± 9.54 ^c	73.96 ± 3.69 ^b
ABB₁₅	7773.3 ± 61.13 ^b	3271.33 ± 34.96 ^d	8324.33 ± 5.77 ^c	3856.67 ± 17.47 ^b	68.17 ± 0.49 ^c
ABB₂₅	7912.67 ± 20.03 ^a	3453.67 ± 4.16 ^c	8448.33 ± 30.24 ^b	3992.67 ± 18.18 ^a	65.34 ± 0.24 ^e
ABC₁₀	7270.67 ± 45.94 ^e	3596.00 ± 114.18 ^c	7269.33 ± 65.19 ^f	3478.00 ± 20.07 ^f	83.24 ± 0.86 ^a
ABC₁₅	7611.33 ± 55.08 ^c	3785.33 ± 56.16 ^b	7374.33 ± 67.63 ^f	3567.67 ± 48.34 ^e	68.83 ± 0.45 ^c
ABC₂₅	8089.33 ± 139.58 ^a	4036.67 ± 87.92 ^a	7691.67 ± 59.20 ^e	3663.33 ± 26.86 ^d	66.85 ± 0.05 ^d

Values are means ± standard deviation, n = 3 ($p < 0.05$). OBP = pure oat bran paste; ABB₁₀, ABB₁₅ and ABB₂₅ = oat bran paste enriched with 10%, 15% and 25% blueberry powder, respectively; ABC₁₀, ABC₁₅ and ABC₂₅ = oat bran paste enriched with 10%, 15% and 25% blackcurrant powder, respectively.

Table 4-2 Pearson's correlations between nutritional components and pasting characteristics

	% Ash	% Moisture	% Total starch	% Protein	PV	Breakdown	FV	Setback	PT
E	0.599	0.804	-0.898*	-0.93**	0.783	0.885*	-0.227	-0.017	-0.578
IDF	0.716*	-0.221	-0.271	-0.181	-0.538	-0.017	-0.472	-0.634	0.502
SDF	0.622	-0.472	-0.594	-0.388	0.693	0.996**	-0.769*	-0.666	-0.58
TDF	0.768*	-0.467	-0.586	-0.384	0.506	0.958**	-0.889**	-0.839*	-0.407
SDF/TDF	0.271	-0.498	-0.451	-0.301	0.789*	0.96**	-0.631	-0.508	-0.711
AUC	-0.12	0.219	-0.337	-0.587	-0.634	-0.893**	0.709	0.667	0.631
% Fat	-0.521	0.128	0.791**	0.893*	-0.215	0.518	-0.88**	-0.925**	0.28
% Ash		-0.108	-0.799**	-0.666**	0.064	0.567	-0.698	-0.645	0.19
% Moisture			0.071	0.123	-0.023	-0.355	0.524	0.64	0.065
% Total starch				0.92**	-0.79*	-0.695	0.259	0.14	0.527
% Protein					-0.828*	-0.806*	0.42	0.283	0.768*
PV						0.675	-0.093	0.02	-0.915**
Breakdown							-0.791*	-0.691	-0.549
FV								0.976**	0.01
Setback									-0.092

* $p < 0.05$, ** $p < 0.01$. Abbreviations: IDF = insoluble dietary fibre; SDF = soluble dietary fibre; TDF = total dietary fibre; PV = peak viscosity; FV = final viscosity; PT = pasting temperature. AUC = area under the curve of the reducing sugar released.



Figure 4.2 Appearance of the pastes

Top row: oat bran paste; middle row: oat bran paste enriched with 10%, 15% and 25% blackcurrant powder, respectively; bottom row: oat bran paste enriched with 10%, 15% and 25% blueberry powder, respectively.

Table 4-3 The colour profiles of pastes

Group	L^*	a^*	b^*	ΔE
OBP	63.37 ± 0.92^a	0.27 ± 0.24^g	12.95 ± 0.49^a	
ABB₁₀	38.69 ± 0.23^b	3.89 ± 0.31^f	-5.79 ± 0.12^d	31.20 ± 0.14^d
ABB₁₅	34.45 ± 0.14^d	5.11 ± 0.48^e	-5.29 ± 0.63^{dc}	34.54 ± 0.47^c
ABB₂₅	29.68 ± 1.01^f	7.08 ± 0.18^d	-7.56 ± 0.30^e	40.03 ± 0.96^b
ABC₁₀	36.70 ± 0.70^c	11.40 ± 0.36^c	-5.81 ± 0.10^d	34.63 ± 0.72^c
ABC₁₅	31.52 ± 0.35^e	16.83 ± 0.37^b	-4.97 ± 0.41^c	40.13 ± 0.30^b
ABC₂₅	25.46 ± 0.28^g	21.11 ± 0.25^a	-1.77 ± 0.11^b	45.10 ± 2.81^a

Values are Mean \pm standard deviation, $n = 3$. Means with different letters within the same column are statistically different ($p < 0.05$). OBP = pure oat bran paste; ABB₁₀, ABB₁₅ and ABB₂₅ = oat bran paste enriched with 10%, 15% and 25% blueberry powder, respectively; ABC₁₀, ABC₁₅ and ABC₂₅ = oat bran paste enriched with 10%, 15% and 25% blackcurrant powder, respectively.

Table 4-4 The Pearson's correlation between TMAC, total starch and protein values and ΔE values

	ΔE
TMAC (extraction)	0.889*
TMAC (gastric)	0.863*
TMAC (intestinal)	0.860*
% protein	-0.930**
%Total starch	-0.898**

* $p < 0.05$, ** $p < 0.01$. TMAC = total monomeric anthocyanidins content.

4.3.3 Nutritional components of raw materials and pastes

Table 4-5 shows the nutritional composition of lyophilised powders of raw materials and pastes. Blackcurrant had the highest total dietary fibre content ($p < 0.01$). Oat bran had the highest fat, protein and starch contents, while the total dietary fibre content accounted for 23% of oat bran ($p < 0.05$). This is consistent with a previous report that oat bran consisted of 16.6% protein, 7.5% fat and 16.5% total dietary fibre (Talukder & Sharma, 2010). It is worth noting that after the pasting procedure (from oat bran to oat bran paste), the soluble dietary fibre content in oat bran paste was 43.05% lower than oat bran ($p < 0.01$). As some dietary fibre may have dissolved in water, this would explain why the ratio of soluble dietary fibre to total dietary fibre decreased in oat bran pastes compared to raw oat bran. Regarding insoluble dietary fibre, the lyophilised powder of oat bran paste contained 20.30% less insoluble dietary fibre than the oat bran ($p < 0.01$). It was noted, during the fibre analysis, the solution of the lyophilised powder of oat bran paste was lighter in colour than the solution of oat bran. This small decrease in insoluble dietary fibre might result from the heating procedure destroying the structure of the insoluble dietary fibre.

Increasing the proportions of powder in the pastes led to a decrease in insoluble dietary fibre content but an increase in soluble dietary fibre. **Table 4-5** illustrates that the insoluble dietary fibre content in 25% blueberry enriched oat bran paste was 9.71% lower compared to oat bran paste ($p < 0.05$), while the soluble dietary fibre content in 25% blueberry and 25% blackcurrant enriched pastes was 4- and 5-fold higher, respectively, than that of the oat bran paste ($p < 0.01$). Interestingly, in terms of the ratio of soluble dietary fibre to insoluble dietary fibre, both blueberry and blackcurrant enriched pastes were much higher than oat bran paste. Samples enriched with 25% blackcurrant showed the highest ratio of soluble dietary fibre to insoluble dietary fibre (61.87%).

The fat content of the oat bran paste was nearly 50% lower than that of oat bran. Positive correlations between fat and protein content ($R = 0.893$; $p < 0.05$) as well as fat and total starch levels ($R = 0.791$; $p < 0.01$) were also found (**Table 4-2**). Hence, it is possible that during the pasting procedure, the binding of lipids to proteins and carbohydrates increased and these bound lipids were not easily extracted with organic solvents. This is in agreement with the results of the starch content in **Table 4-5**, which showed that compared with the oat bran paste, blueberry and blackcurrant enriched pastes contained less starch, especially at 25% supplementation, which were 20.24% and 39.99% lower, respectively ($p < 0.01$).

Table 4-5 The Nutritional components of raw materials and pastes

Group	Fat	Ash	Moisture	Total starch	Protein	Dietary fibre			
						IDF	SDF	TDF	SDF: TDF
	-----% (w/w) -----								
Raw Materials									
Oat bran	7.91 ± 0.18 ^A	1.99 ± 0.56 ^B	2.34 ± 0.22 ^A	45.66 ± 0.18 ^A	13.10 ± 0.17 ^A	16.05 ± 0.60 ^B	6.95 ± 0.24 ^C	23.00 ± 0.21 ^B	30.22
Blueberry	0.91 ± 0.12 ^B	1.67 ± 0.07 ^B	1.63 ± 0.10 ^B	23.60 ± 0.07 ^B	4.23 ± 0.09 ^B	10.81 ± 0.11 ^C	8.71 ± 0.45 ^B	19.52 ± 0.55 ^C	44.59
Blackcurrant	0.66 ± 0.13 ^B	6.10 ± 0.99 ^A	1.45 ± 0.06 ^B	4.26 ± 0.24 ^C	2.85 ± 0.13 ^C	19.12 ± 0.92 ^A	22.45 ± 0.40 ^A	41.57 ± 1.31 ^A	54.03
Pastes									
OBP	4.03 ± 0.01 ^e	1.98 ± 0.11 ^c	2.14 ± 0.06 ^a	47.14 ± 0.16 ^b	12.71 ± 0.14 ^a	12.36 ± 0.14 ^c	3.96 ± 0.10 ^f	16.32 ± 0.34 ^d	24.26
ABB₁₀	5.26 ± 0.04 ^b	1.78 ± 0.04 ^d	1.13 ± 0.09 ^d	47.76 ± 0.00 ^a	12.65 ± 0.06 ^a	15.27 ± 0.23 ^a	10.33 ± 0.30 ^e	25.60 ± 0.53 ^c	40.34
ABB₁₅	4.95 ± 0.12 ^c	1.70 ± 0.05 ^d	1.15 ± 0.02 ^d	46.38 ± 0.03 ^c	11.97 ± 0.10 ^b	13.97 ± 0.35 ^b	11.90 ± 0.59 ^d	25.87 ± 0.94 ^c	46.00
ABB₂₅	4.45 ± 0.02 ^d	1.79 ± 0.08 ^{cd}	1.81 ± 0.18 ^b	37.60 ± 0.04 ^e	11.75 ± 0.16 ^b	11.16 ± 0.23 ^d	14.93 ± 0.40 ^c	26.09 ± 0.63 ^c	57.21
ABC₁₀	6.73 ± 0.31 ^a	2.23 ± 0.13 ^{ab}	1.16 ± 0.04 ^d	44.08 ± 0.13 ^d	12.55 ± 0.08 ^a	15.83 ± 0.28 ^a	15.22 ± 0.30 ^c	31.04 ± 0.58 ^b	49.02
ABC₁₅	5.58 ± 0.39 ^b	2.15 ± 0.06 ^{bc}	1.51 ± 0.04 ^c	43.94 ± 0.19 ^d	11.14 ± 0.08 ^c	13.42 ± 0.25 ^b	17.60 ± 0.23 ^b	31.02 ± 0.02 ^b	56.73
ABC₂₅	5.03 ± 0.08 ^c	2.36 ± 0.04 ^a	1.57 ± 0.06 ^c	28.29 ± 0.29 ^f	10.84 ± 0.09 ^d	12.34 ± 0.28 ^c	20.02 ± 0.42 ^a	32.36 ± 0.71 ^a	61.87

Values are Mean ± standard deviation, n = 3. Means with different letters within the same column are statistically different ($p < 0.05$). IDF = insoluble dietary fibre; SDF = soluble dietary fibre; TDF = total dietary fibre. All values are based on dry basis. OBP = pure oat bran paste; ABB₁₀, ABB₁₅ and ABB₂₅ = oat bran paste enriched with 10%, 15% and 25% blueberry powder, respectively; ABC₁₀, ABC₁₅ and ABC₂₅ = oat bran paste enriched with 10%, 15% and 25% blackcurrant powder, respectively.

4.3.4 The major phenolic acids and anthocyanins in extracts

In this study, twelve phenolic compounds and their retention time (**Table 4-6**) were characterised at 280 nm (**Fig. 4-3**), 320 nm (**Fig. 4-4**), and 360 nm (**Fig. 4-5**), respectively. The results are shown in **Table 4-7**, it was not surprising that the extracts from blueberry and blackcurrant powder, and pastes contained significantly more phenolic acids compared to oat bran and paste ($p < 0.01$). Apart from gallic acid, rutin was the major phenolic compound in the extracts of blueberry and blackcurrant powders, and pastes between 280 nm and 360 nm, and its derivatives have also been reported as a major antioxidant among bioactive compounds (Zhang, Jia, Ma, Cheng, & Cai, 2018).

Four anthocyanins, including delphinidin (0.229 ± 0.004 mg/g sample), cyanidin (0.137 ± 0.002 mg/g sample), petunidin (0.143 ± 0.002 mg/g sample), and malvidin (0.378 ± 0.005 mg/g sample) were detected at 520 nm in the extract of 25% blueberry enriched paste, while two anthocyanins, delphinidin (0.262 ± 0.003 mg/g sample), and cyanidin (0.456 ± 0.003 mg/g sample) were identified in the extract of 25% blackcurrant enriched paste (**Fig. 4-6**). Malvidin accounted for the highest content in the extract of 25% blueberry enriched paste, followed by delphinidin. By contrast, the content of cyanidin was 1.5-fold higher than the content of delphinidin in the extract of 25% blackcurrant enriched paste. This was in agreement with a previous study (Z. Diaconeasa, L. Leopold, D. Rugină, H. Ayvaz, & C. Socaciu, 2015), reporting that the major anthocyanins in blueberry and blackcurrant juice extracts were delphinidin, cyanidin, petunidin, and malvidin. However, their results showed higher anthocyanin content compared to the results shown in this thesis. One reason for this observation may be because the extracts they used were in the form of the purified juice, while in this thesis, the food matrix effects, in particular of the other nutritional components surrounded by phenolic compounds were considered to affect the functional properties.

Table 4-6 The Phenolic compounds and their retention time

Retention time (min)	Quantification wavelength (nm)	Compound name
10.407	280 nm	Gallic acid
14.78	280 nm	Protocatechuic acid
19.7	320 nm	Caftaric acid
20.0	280 nm	Hydrobenzoric acid
22.9	280 nm	Catechin
24.89	320 nm	Caffeic acid
25.29	280 nm	Syringic acid
27.3	280 nm	Epicatechin
31.509	320 nm	p-Coumaric acid
35.4	320 nm	Ferulic acid
37.0	360 nm	Rutin
62.3	360 nm	Quercetin
20	520 nm	Delphinidin
22.5	520 nm	Cyanidin
23.5	520 nm	Petunidin
25.8	520 nm	Malvidin

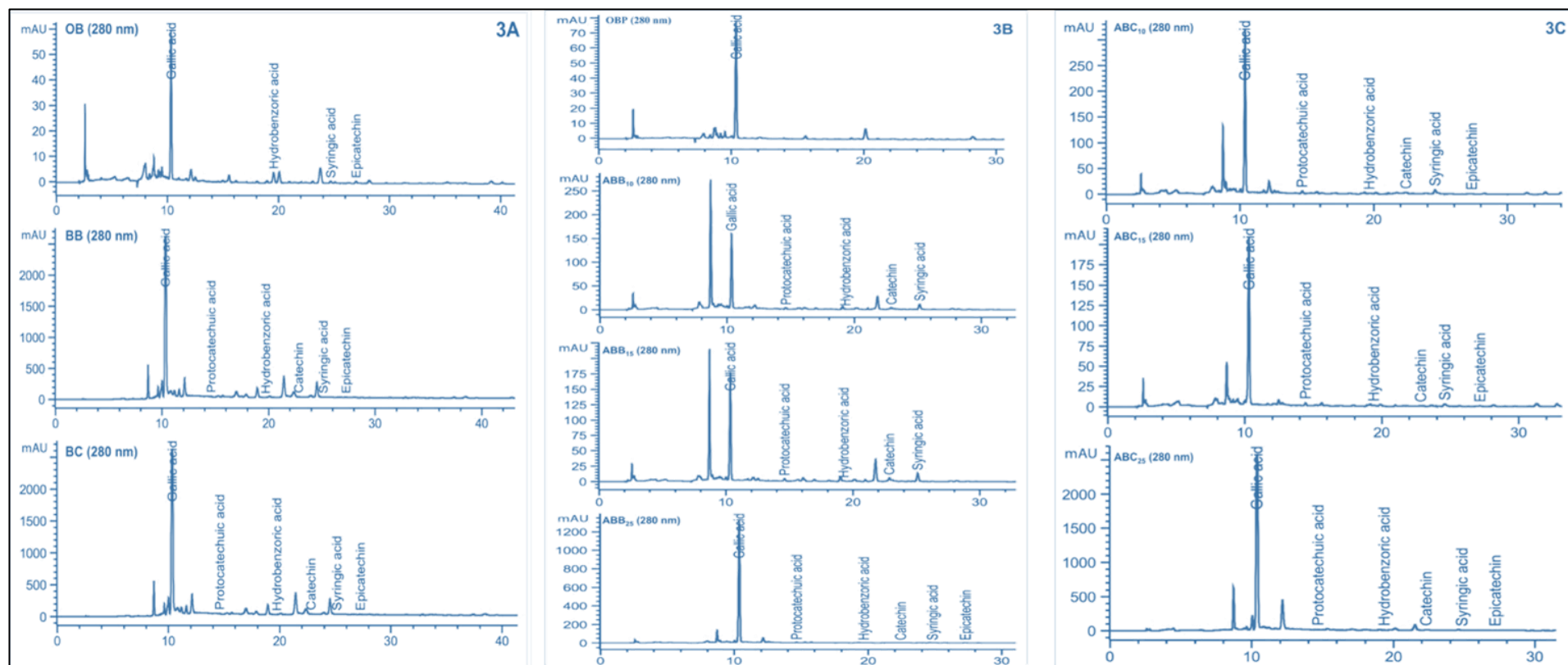


Figure 4.3 Captions HPLC chromatograms of phenolic acids in extracts at 280 nm

(3A): raw materials; (3B): oat bran paste and blueberry enriched paste; (3C): blackcurrant enriched paste.

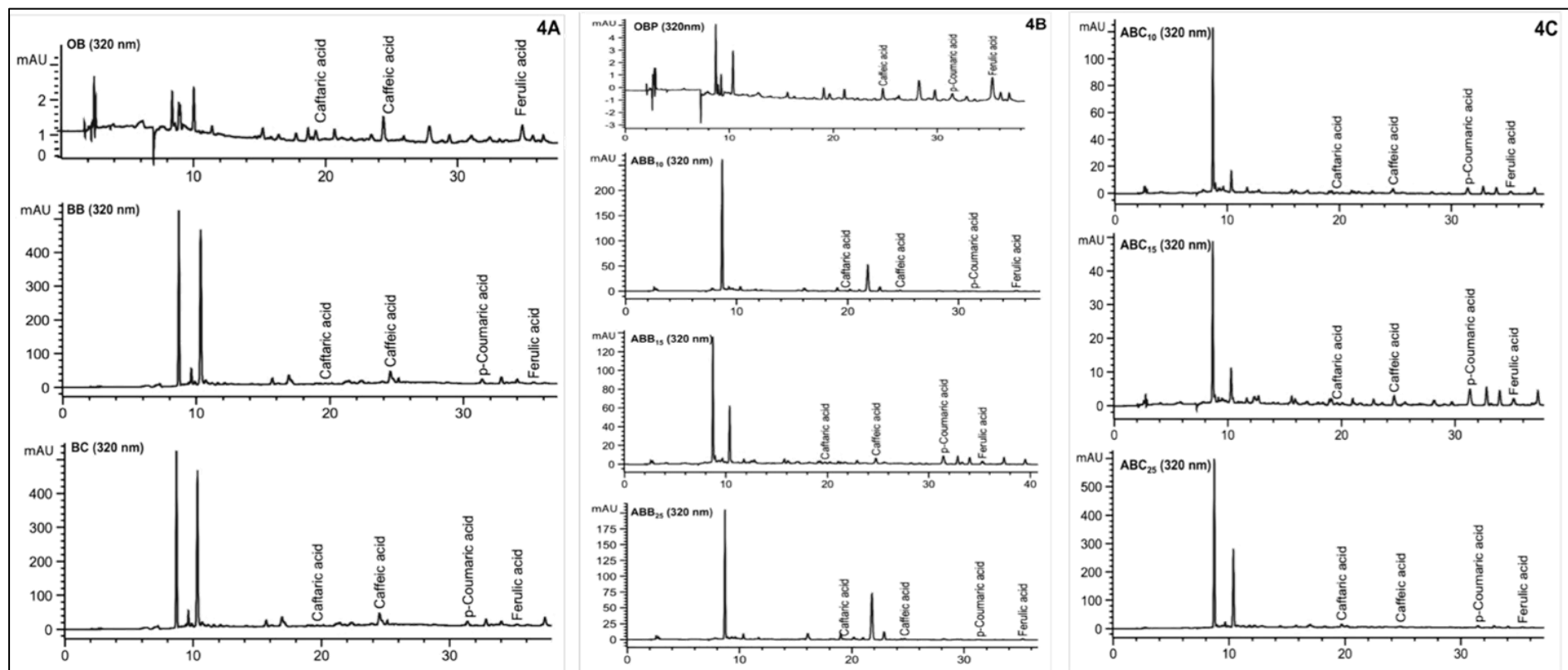


Figure 4.4 Captions HPLC chromatograms of phenolic acids in extracts at 320 nm

(4A): raw materials; (4B): oat bran paste and blueberry enriched paste; (4C): blackcurrant enriched past

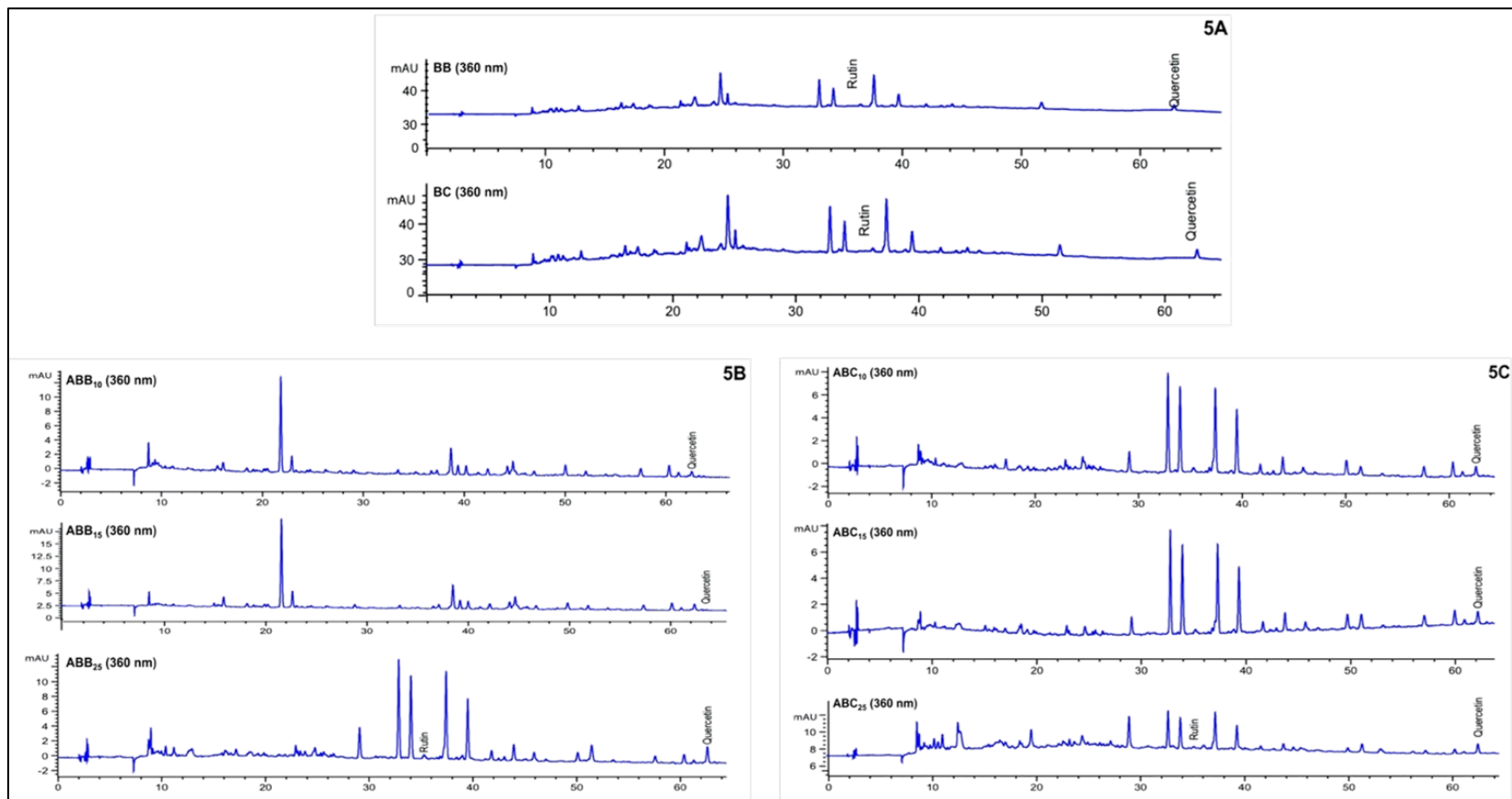


Figure 4.5 Captions HPLC chromatograms of phenolic acids in extracts at 360 nm

(5A): raw materials; (5B): oat bran paste and blueberry enriched paste; (5C): blackcurrant enriched paste.

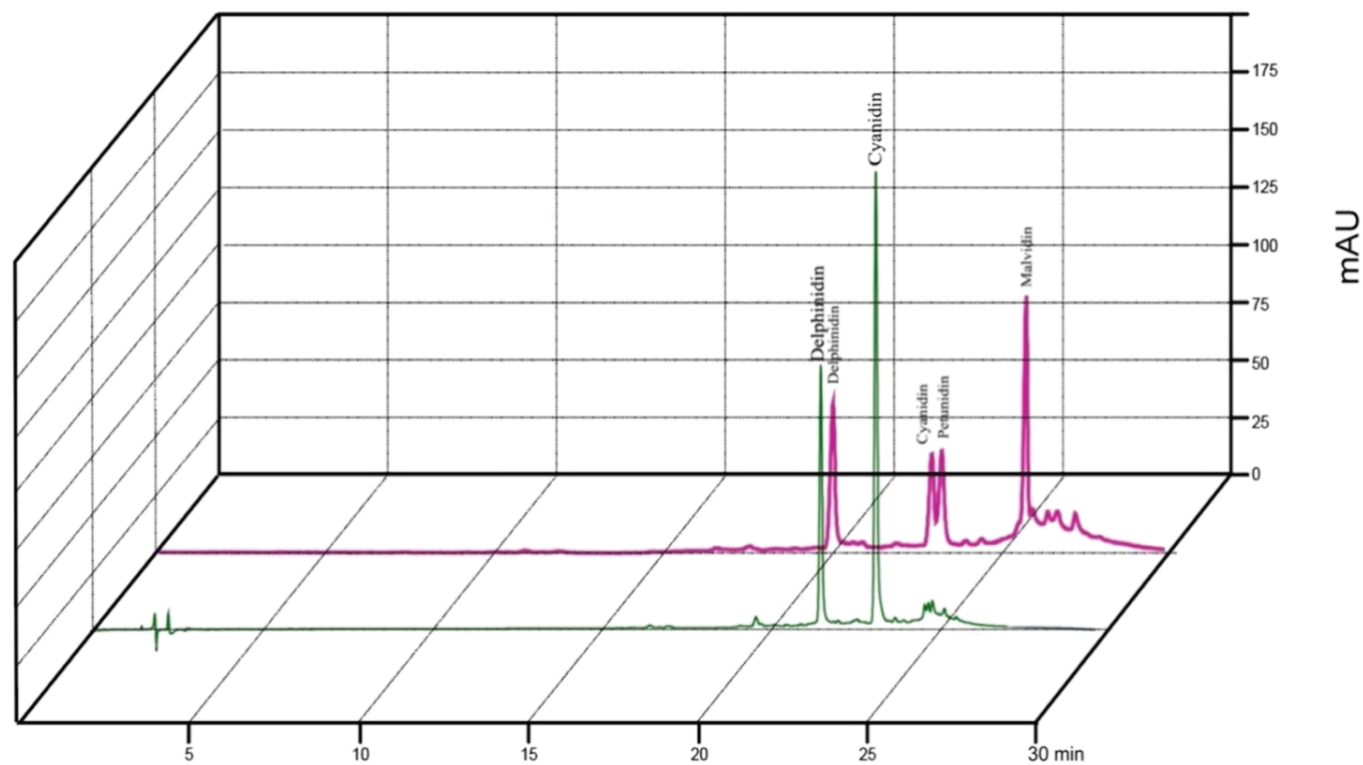


Figure 4.6 HPLC chromatograms of anthocyanins of ABB₂₅ and ABC₂₅ at 520 nm.

ABB₂₅ = 25% blueberry enriched oat bran paste; ABC₂₅ = 25% blackcurrant enriched oat bran paste

Table 4-7 The major phenolic compounds content determined by HPLC (µg/100 g sample)

Group	Gallic acid	Caffeic acid	Quercetin	Ferulic acid	Syringic acid	Rutin	Protocatechuic acid	Hydrobenzoic acid	p-Coumaric acid	Catechin	Epicatechin	Caftaric acid
Oat bran	60.24 ± 3.74 ^C	8.39 ± 0.18 ^C	ND	0.38 ± 0.05 ^C	2.36 ± 0.26 ^C	ND	ND	0.68 ± 0.08 ^C	ND	ND	3.50 ± 0.25 ^C	2.70 ± 0.26 ^C
Blueberry	2702.76 ± 75.06 ^A	59.59 ± 2.53 ^B	13.56 ± 0.69 ^A	10.57 ± 0.76 ^B	390.56 ± 19.28 ^A	6.73 ± 0.70 ^B	22.58 ± 1.01 ^B	21.92 ± 1.06 ^B	19.80 ± 1.38 ^A	100.45 ± 1.82 ^B	22.99 ± 1.20 ^B	16.59 ± 1.12 ^A
Blackcurrant	2537.69 ± 54.07 ^B	83.54 ± 0.92 ^A	10.32 ± 0.20 ^B	13.57 ± 0.89 ^A	322.46 ± 22.19 ^B	8.36 ± 0.09 ^A	31.35 ± 1.44 ^A	27.04 ± 3.20 ^A	15.42 ± 0.72 ^B	136.86 ± 8.00 ^A	45.54 ± 2.08 ^A	12.20 ± 1.01 ^B
OBP	8.22 ± 0.37 ^B	0.04 ± 0.00 ^B	ND	0.15 ± 0.03 ^B	ND	ND	ND	ND	0.03 ± 0.00 ^B	ND	ND	ND
ABB₁₀	12.67 ± 0.34	0.25 ± 0.01 ^e	0.22 ± 0.01 ^{cd}	0.90 ± 0.12 ^e	1.76 ± 0.12 ^c	ND	0.54 ± 0.02 ^c	1.16 ± 0.07 ^B	0.74 ± 0.10 ^f	1.03 ± 0.04 ^e	ND	0.99 ± 0.05 ^e
ABB₁₅	15.19 ± 0.23 ^c	0.45 ± 0.02 ^d	0.25 ± 0.02 ^b	2.13 ± 0.07 ^d	3.59 ± 0.15 ^b	ND	1.30 ± 0.05 ^b	2.25 ± 0.12 ^B	1.38 ± 0.05 ^d	1.47 ± 0.12 ^c	ND	1.49 ± 0.05 ^c
ABB₂₅	234.74 ± 6.06 ^b	0.75 ± 0.03 ^b	0.53 ± 0.01 ^a	3.49 ± 0.18 ^a	6.12 ± 0.39 ^a	ND	2.34 ± 0.06 ^a	3.27 ± 0.08 ^a	2.09 ± 0.05 ^c	2.84 ± 0.08 ^b	0.96 ± 0.06 ^b	3.02 ± 0.84 ^b
ABC₁₀	34.24 ± 1.22 ^f	0.47 ± 0.03 ^d	0.05 ± 0.00 ^d	0.47 ± 0.02 ^{cd}	1.18 ± 0.07 ^c	ND	1.00 ± 0.06 ^d	0.64 ± 0.03 ^B	0.55 ± 0.02 ^e	0.76 ± 0.15 ^f	0.50 ± 0.02 ^f	0.96 ± 0.13 ^f
ABC₁₅	248.63 ± 13.34 ^d	0.67 ± 0.04 ^c	0.26 ± 0.01 ^c	0.90 ± 0.05 ^c	2.38 ± 0.05 ^b	0.20 ± 0.02 ^b	5.09 ± 0.15 ^b	2.38 ± 0.46 ^B	1.23 ± 0.08 ^b	1.83 ± 0.13 ^d	1.08 ± 0.03 ^d	1.75 ± 0.08 ^d
ABC₂₅	249.04 ± 5.03 ^a	1.06 ± 0.04 ^a	0.48 ± 0.05 ^b	1.44 ± 0.11 ^b	3.06 ± 0.07 ^b	0.30 ± 0.01 ^a	8.05 ± 0.41 ^a	3.81 ± 0.15 ^b	1.97 ± 0.11 ^a	2.45 ± 0.08 ^a	1.59 ± 0.15 ^a	2.55 ± 1.00 ^a

Values are mean ± standard deviation, n = 3. Raw materials and pastes were compared separately. Comparison within the same row is expressed by upper case letters, while comparison within the same column is expressed by lower case letters ($p < 0.05$). All values are based on dry basis. OBP = pure oat bran paste; ABB₁₀, ABB₁₅ and ABB₂₅ = oat bran paste enriched with 10%, 15% and 25% blueberry powder, respectively; ABC₁₀, ABC₁₅ and ABC₂₅ = oat bran paste enriched with 10%, 15% and 25% blackcurrant powder, respectively; ND = no data.

4.3.5 The total monomeric anthocyanin contents (TMAC) in extracts

Anthocyanin pigment experiences a reversible structural transformation as a result of changes in pH. This can alter the absorption spectrum, thus the pH differential method measures the TMAC values rapidly and accurately, even when degraded polymerised pigments and other interfering compounds are present (Khoo *et al.*, 2017). **Table 4-8** shows the change of TMAC during the *in vitro* digestion. Among all of the undigested extracts, blackcurrant powder extract had the highest TMAC (36.27 mg Cy-3GE/100 g), followed by blueberry powder extract (14.96 mg Cy-3GE/100 g). TMAC in oat bran extract was low, 0.03 mg Cy-3GE/100 g. In terms of the extracts of pastes, the TMAC in the extract of pure oat bran paste was too low to be detected. TMAC in the extracts of blueberry and blackcurrant enriched pastes varied from 0.25 to 0.66 mg Cy-3GE/100 g. The extract of blackcurrant enriched paste was given more TMAC compared with the corresponding extract of blueberry enriched paste ($p < 0.01$). TMAC in all extracts decreased after undergoing the *in vitro* digestion. TMAC in the extracts of blueberry and blackcurrant powder decreased over 95% compared to the corresponding undigested extracts ($p < 0.01$). TMAC in oat bran extract could not be detected in both the gastric and intestinal phase. The chemical reactivity of anthocyanins is also essential in understanding their fate after digestion and their impact on health as anthocyanins may be digested as a complex mixture of native forms, derivatives, and degradation products that allow themselves to be involved in the digestive tract (Dangles & Fenger, 2018). These results could be responsible for the change of pH. As a food colourant, anthocyanin with a lower pH (acidic conditions) has a significant effect, which gives a red pigment (Wahyuningsih, Wulandari, Wartono, Munawaroh, & Ramelan, 2017). A logarithmic relationship has been identified between the temperature and the rate of anthocyanin degradation. Higher temperatures promote the transition to the unstable form of the chalcone, whereas the opened C-ring of the chalcone is further degraded to brown products (Deng *et al.*, 2019).

Refrigeration is an effective means of preserving anthocyanins. Muche, Speers, and Rupasinghe (2018) compared with the amount of anthocyanidin content lost in blackcurrant juice stored at 4 °C and 37 °C, observing that blackcurrant anthocyanidin contents lost only 40% at 4 °C, while no measurable amounts of anthocyanidin were found at 37 °C. It is worth noting that short-term high temperature treatment could improve the stability of anthocyanin by facilitating the inactivation of native enzymes that are harmful to anthocyanins. Heat-assisted drying processes decompose anthocyanins considerably (Waterhouse, Sun-Waterhouse, Su, Zhao, & Zhao, 2017), not only due to the high temperature, but also owing to the drying process, which increases the concentration of the reagents in the solution (Loypimai, Moongngarm, & Chottanom, 2016). The freeze drying technique has been widely used in high-quality food preparation since this technique can preserve the bioactive compounds during the drying process (Silva-Espinoza, Ayed, Foster, Camacho, & Martínez-Navarrete, 2019). The results from this chapter illustrate that the blueberry and blackcurrant enriched pastes experienced a short-term high temperature procedure (pasting process, 95 °C for 10 min), and afterwards they were treated with freeze drying. The decline of the extracts from blueberry and blackcurrant enriched pastes was much smaller than the extracts from raw materials. Even though all the extracts from pastes experienced the decrease after the *in vitro* digestion, the extract of blackcurrant enriched paste exhibited a higher TMAC value compared with corresponding extract of blueberry enriched paste, in particular of the extract from 25% blackcurrant enriched paste, which still had the highest TMAC (0.35 mg Cy-3GE/g) among all extracts of digested pastes ($p < 0.01$). Generally, changes in TMAC were influenced by the combined effects of the types of dietary supplements, and the phases of the *in vitro* digestion. These effects were considered extremely significant ($p < 0.01$). According to the percentage of total variation, the different sources and proportions of supplements were the main reasons for the variation, indicating that the anthocyanins from different food sources

could be influenced by their food matrix effects on their chemical structures and stability as well as their bioavailability.

4.3.6 *In vitro* starch digestibility and area under the curve (AUC)

Fig. 4-7A & 4-7B display the amount of reducing sugar released over a 120-min during an *in vitro* digestion of raw materials and pastes, respectively. The standardised AUC values of both raw materials and paste is shown in **Fig. 4-7C**. Blueberry and blackcurrant enriched pastes released much less reducing sugar during the *in vitro* digestion procedure, compared to that of oat bran paste ($p < 0.01$). In terms of the raw materials, blueberry powder released the least reducing sugar compared to blueberry and oat bran ($p < 0.05$). Starch can be graded as rapidly digested starch, slowly digested starch, and resistant starch. The slowly digested starch fraction is digested after the rapidly digested starch (Chung, Liu, & Hoover, 2009). All samples showed a dramatically increased rate of reducing sugars release in the first 20 min of digestion (the RDS fraction), when compared to the other time periods during the digestion, with the peak values being reached at 20 min or 60 min. There was a significant difference between oat bran paste and blueberry enriched paste as well as blackcurrant enriched paste ($p < 0.01$). For blueberry and blackcurrant enriched pastes, between 20 and 120 min, the slope of the digestion curves flattened out, compared to the curves at 0 - 20 min. By contrast, the pure oat bran paste still showed an upward trend between 20 and 60 min before levelling off. A similar pattern in digestion curves was observed of raw materials, blueberry and blackcurrant powder generated an rising values during the first 20-min digestion. After 20 min, the slope of the cruves began to flatten off, while the digestion curve of oat bran displayed signs of levelling off after 60 min. The supplements of blueberry and blackcurrant powders decreased the reducing sugars released. As shown in **Fig. 4-7C**, the AUC value of blueberry and blackcurrant enriched oat bran pastes dropped by approximately 50% compared to the oat bran pastes.

However, there were no significant differences in the AUC amongst the different concentrations (10-25%) of blueberry or blackcurrant powders added.

Blueberry and blackcurrant powders contain large amounts of fibre and antioxidants, which could be responsible for the lower AUC of blueberry and blackcurrant enriched pastes. Increasing levels of supplements also increased the total dietary fibre and TMAC values. Increased dietary fibre levels can reduce the rate of starch digestion, leading to a reduction in postprandial glycaemia since dietary fibre inhibits digestive enzymes such as α -amylase and α -glucosidase (McDougall *et al.*, 2005). Phenolics can also inhibit enzyme activities through competitive, non-competitive or uncompetitive mechanisms. A previous study from Yazdankhah, Hojjati, and Azizi (2019) revealed that pasta supplemented with 1.5% berry extract could result in a significantly lower GI through the inhibition of enzyme activity. Generally, phenolics are very sensitive to alkaline solutions and can produce reactive species in alkaline conditions. Once these phenolics react with the free amino acid groups of digestive enzymes, the physicochemical properties of these phenolics will change. During the *in vitro* digestion, non-covalent interactions, such as hydrogen bonding, hydrophobic and ionic interactions may occur between the phenolics and starch molecules, which may in turn affect the starch degradation (Bordenave, Hamaker, & Ferruzzi, 2014).

4.4 Conclusion

Enrichment of oat bran paste with blackcurrant or blueberry powder significantly changed the pasting properties and colour characteristics. Blueberry and blackcurrant enriched oat bran paste had increased the amounts of phenolic compounds as well as decreased the reducing sugar release during the *in vitro* digestion. Such observations demonstrated the potential for enhanced antioxidant activity and lowered predictive glycaemic response through the integration of blueberry or blackcurrant powder with oat bran, which could be

investigated further for future formulations of food and nutraceutical health-promoting supplement.

Table 4-8 TMAC in extracts of raw materials and pastes

TMAC (mg Cy-3GE/100g sample)						
Group	<i>In vitro</i> digestion phase			Samples (A)	<i>Total variation</i>	
	Extraction	Gastric	Intestinal		Digestion phase (B)	A*B
Oat bran	0.03 ± 0.01 ^{Ac}	ND	ND			
Blueberry	14.96 ± 1.48 ^{Ab}	0.99 ± 0.03 ^{Bb}	0.62 ± 0.02 ^{Cb}	88.10% (p < 0.001)	7.34% (p < 0.001)	4.53% (p < 0.001)
Blackcurrant	36.27 ± 1.69 ^{Aa}	3.90 ± 0.03 ^{Ba}	3.43 ± 0.04 ^{Ca}			
OBP	ND	ND	ND			
ABB ₁₀	0.25 ± 0.02 ^{Al}	0.11 ± 0.02 ^{Bk}	ND			
ABB ₁₅	0.30 ± 0.01 ^{Ak}	0.13 ± 0.02 ^{Bk}	0.04 ± 0.01 ^{Ck}			
ABB ₂₅	0.41 ± 0.00 ^{Aj}	0.16 ± 0.02 ^{Bj}	0.08 ± 0.00 ^{Cj}	71.70% (p < 0.001)	18.40% (p < 0.001)	9.16% (p < 0.001)
ABC ₁₀	0.40 ± 0.01 ^{Ai}	0.18 ± 0.03 ^{Bj}	0.12 ± 0.01 ^{Ci}			
ABC ₁₅	0.64 ± 0.00 ^{Ah}	0.40 ± 0.03 ^{Bi}	0.13 ± 0.02 ^{Ci}			
ABC ₂₅	0.66 ± 0.03 ^{Ah}	0.62 ± 0.02 ^{Bh}	0.35 ± 0.01 ^{Ch}			

Values are mean ± standard deviation, n = 3. Raw materials and pastes were compared separately. Values with different uppercase letters, in the same row, are statistically different ($p < 0.05$), while values with different small case letters, in the same column, are statistically different ($p < 0.05$). TMAC = total monomeric anthocyanins content; OBP = pure oat bran paste; ABB₁₀, ABB₁₅ and ABB₂₅ = oat bran paste enriched with 10%, 15% and 25% blueberry powder, respectively; ABC₁₀, ABC₁₅ and ABC₂₅ = oat bran paste enriched with 10%, 15% and 25% blackcurrant powder, respectively; ND = no data. All values are based on dry basis.

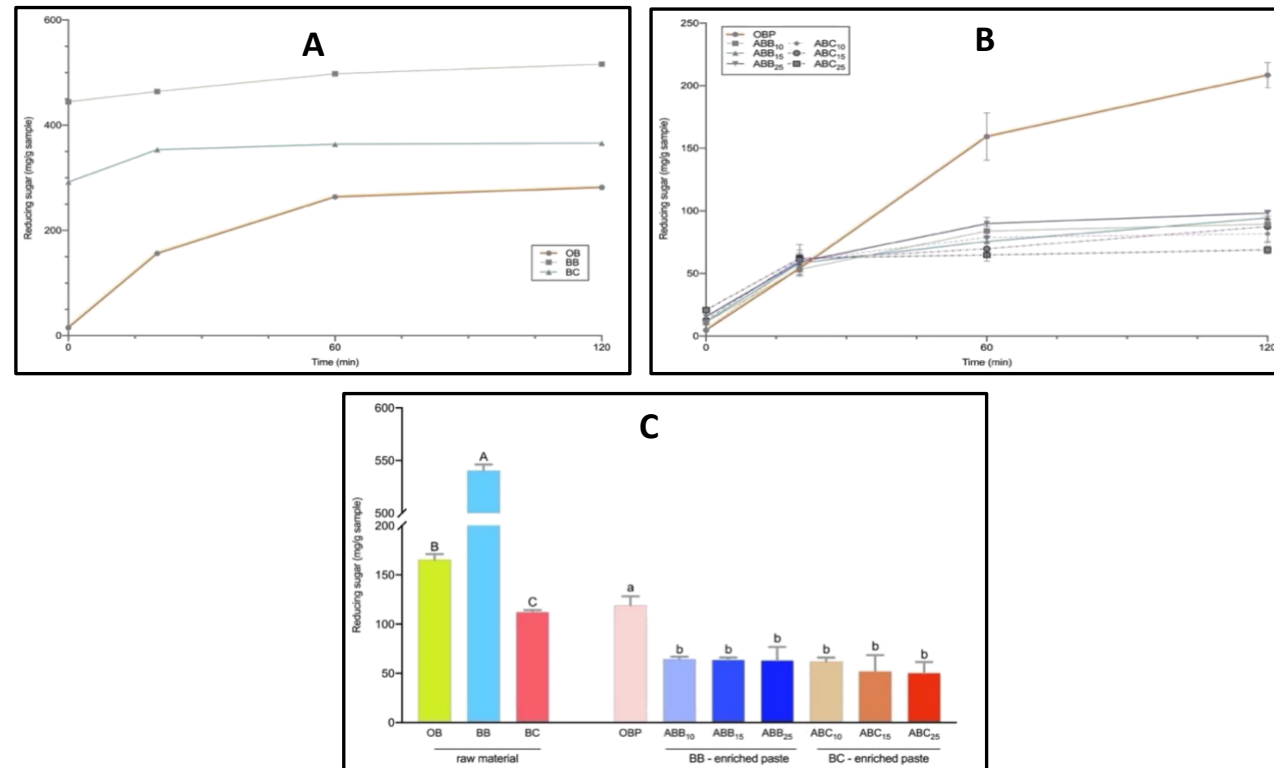


Figure 4.7 Reducing sugar released during the in vitro digestion

(A): reducing sugar released of raw materials; (B): reducing sugar released of pastes; (C): Values of reducing sugar during a 120-min *in vitro* digestion for area under the curve (AUC). Error bars represent standard deviation of replicates. The different letter means there is difference between each other ($p < 0.05$). OBP = pure oat bran paste; ABB10, ABB15 and ABB25 = oat bran paste enriched with 10%, 15% and 25% blueberry powder, respectively; ABC10, ABC15 and ABC25 = oat bran paste enriched with 10%, 15% and 25% of blackcurrant powder, respectively.

Chapter 5

The bioactive ingredients from blueberry and blackcurrant powder enhance the antioxidant capacity of oat bran paste

Abstract

In this chapter, oat bran was enriched with 0%, 10%, 15% and 25% (w/w) of blueberry and blackcurrant powder, respectively, and the Rapid Viscosity Analyser (RVA) was used to form the blueberry and blackcurrant enriched pastes. The combination of an *in vitro* digestion process with three antioxidant assays was employed to observe the effects of blueberry and blackcurrant powder on the changes of phenolic compounds and the antioxidant potential of oat bran paste. Cyanidin, delphinidin, malvidin, pelargonidin, and peonidin were identified by pH differential method. The results showed that the blueberry and blackcurrant enriched pastes increased the total phenolic content and the antioxidant capacity of oat bran paste, while the total flavonoid content decreased after the digestion. Strong correlations between these bioactive compounds and antioxidant values were also observed. This chapter found that the bioactive ingredients from the blueberry and blackcurrant powder enhanced the antioxidant capacity of oat bran paste, and oat bran pastes enriched with blueberry and blackcurrant powder have the potential to be utilised in the development of the functional foods.

Keywords: *in vitro* digestion; anthocyanidin; antioxidant; phenolic compounds

5.1 Introduction

Obesity is a global social problem, and it can lead to several diseases, such as type 2 diabetes mellitus (T2DM), cardiovascular diseases, and cancer (Hruby & Hu, 2015). Obesity is identified by an increase in body weight resulting from excessive fat accumulation, and is also characterised by chronic low-grade inflammation with permanently increased oxidative stress (Marseglia *et al.*, 2015). Over-expression of oxidative stress damages cellular structures along with under-production of antioxidant mechanisms, leading to the progression of obese-related complications (Sharifi-Rad *et al.*, 2020) (Manna & Jain, 2015). Therefore, the consumption of antioxidant molecules has been shown to be effective as a strategy to prevent, or reduce, the risk of these diseases (Sarangarajan, Meera, Rukkumani, Sankar, & Anuradha, 2017).

Research has indicated benefits of natural antioxidants, (including being safer, and higher values for anti-inflammatory properties), compared with synthetic antioxidants, however, the latter are more stable and multifunctional (Brglez Mojzer, Knez Hrnčič, Škerget, Knez, & Bren, 2016). Polyphenols have been extensively studied in cell culture and animal studies for their protective role (Cory *et al.*, 2018). As mentioned in Chapter 4, blueberry and blackcurrant contain a large number of polyphenols, with antioxidants properties. The phenolic groups in polyphenols can accept the formation of relatively stable phenoxyl radicals by the electron, thereby disrupting the oxidation chain reactions in cellular components (Copetti *et al.*, 2018). Polyphenol rich foods have been found to protect cells from oxidative stress and to improve the ability of plasma antioxidants, thus reducing the risk of different human chronic diseases, including T2DM (Dal & Sigrist, 2016). This increase in plasma antioxidant capacity following the consumption of polyphenol-rich food may be responsible either for the presence of polyphenols and their metabolites in plasma, or for their preservative effects on other

reducing agents, such as endogenous antioxidants, or for their effect on the absorption of pro-oxidative food components, such as iron (Kalt *et al.*, 2019; Tan, Norhaizan, Liew, & Sulaiman Rahman, 2018).

Oat bran is particularly high in antioxidants compared to other parts of the oat grain. Recent studies have also shown that the health benefit of oats is mainly due to the antioxidants found in the bran along with phenolic compounds, such as potent avenanthramides, which are a family of antioxidants unique to oats (Kristek *et al.*, 2018; Tan *et al.*, 2018). Avenanthramide has been demonstrated to be linked to the potential of reducing inflammation, being anticancer, and lowering blood sugar levels (Bratt *et al.*, 2003). A concentrated extract of oat bran could be used as a natural preservative for foods rich in unsaturated long-chain fatty acids since oat bran is a good source of antioxidants. A concentrated extract of oat bran may protect these long-chain fatty acids from oxidative stress, and from creating off-flavours in foods (Duță, Culețu, & Mohan, 2018).

Berries have a positive image and their inclusion in cereal products appears to be a promising strategy to enhance their antioxidant capacity (Ma, Sun, Zeng, Luo, & Yang, 2018). However, few studies have reported the use of combinations of berry fruits with oat bran (Cebeci & Şahin-Yeşilçubuk, 2014), and no study has focused on the potential synergistic effect on the food matrix of combining berries and oat bran.

Chapter 4 reported that the bioactive compounds, especially anthocyanins from blueberry and blackcurrant powders altered the physicochemical and nutritional properties of oat bran paste, and the predicted hypoglycaemic properties. This chapter focuses on the effect that these bioactive compounds, blueberry and blackcurrant powder have, on the changes of antioxidant capacity of oat bran paste during the *in vitro* digestion. Additionally, the pH differential method was employed to identify the major anthocyanidins in blueberry and blackcurrant enriched pastes.

5.2 Methods

5.2.1 Preparation of oat bran paste

Pastes were prepared as described in 3.2.

5.2.2 Simulation of the *in vitro* digestion

The *in vitro* digestion is described in 3.6.

5.2.3 Extraction of raw materials and pastes

The extraction of both undigested and digested samples was carried out as described in 3.5.

5.2.4 Total phenolic content (TPC) in extracts

TPC was determined using Folin-Ciocalteu reagent as illustrated in 3.9.

5.2.5 Total flavonoid content (TFC) in extracts

TFC was determined using aluminium chloride reagent as outlined in 3.10.

5.2.6 Identification of the major anthocyanidin content in extracts

The major anthocyanidin content of extracts from undigested and digested samples was determined by the pH differential method according to the method outlined in 3.11.

5.2.7 DPPH assay

The radical scavenging capacity of extracts of undigested and digested samples, were determined by the DPPH assay as described in 3.13.1.

5.2.8 ABTS assay

The ABTS assay was carried out as outlined in 3.13.2.

5.2.9 FRAP assay

The reducing of antioxidant power activity of extracts from undigested and digested samples was determined by using FRAP reagent solution as described in 3.13.3.

5.2.10 Statistical analysis

Statistical analysis was carried out as outlined in 3.17.

5.3 Results and discussion

5.3.1 Changes in TPC and TFC during *in vitro* digestion

Table 5-1 shows the TPC in extracts of raw materials and pastes at different digestion phases.

Overall, although there was a decline in the TPC content in both raw materials and pastes after intestinal digestion, compared with the TPC after gastric digestion, the polyphenol concentration of each of the digested samples was higher than that of the corresponding undigested sample ($p < 0.01$ or $p < 0.05$). Before digestion, blackcurrant powder yielded the highest TPC value of 97.15 mg GAE/g, followed by blueberry powder (84.91 mg GAE/g), while oat bran paste showed the lowest TPC value of 0.43 mg GAE/g. All of the blackcurrant enriched pastes had higher TPC values than those of the blueberry enriched pastes. The difference became more evident as the level of blueberry or blackcurrant powder increased in the pastes. The TPC in the extract of undigested 25% blackcurrant enriched paste was 10-fold higher than that of undigested 25% blueberry enriched paste and the ratio reduced to 2.4-fold after the intestinal digestion, thus 25% blackcurrant enriched paste had the highest TPC value ($p < 0.01$). The TFC of the samples (**Table 5-2**) illustrated that blueberry powder displayed the highest TFC across the whole digestion, while oat bran had the lowest TFC value. The TFC value of all samples firstly increased after the gastric digestion, then decreased after the intestinal digestion. In oat bran, the TFC value after the intestinal digestion was so low, it could not be detected. Blueberry enriched pastes exhibited higher levels of TFC than that of blackcurrant

enriched pastes. For instance, before digestion, the TFC value of 25% blueberry enriched paste was 45.12% higher than that of 25% blackcurrant enriched pastes ($p < 0.01$). After the intestinal digestion, the TFC of 25% blueberry enriched paste was 30% lower than the corresponding undigested sample, although it was still 9.4% higher than that of the intestinal digesta of 25% blackcurrant enriched pastes ($p < 0.01$). The TFC trend of blueberry powder > blackcurrant powder > blueberry enriched paste > blackcurrant enriched paste > oat bran > oat bran paste was maintained for TFC throughout the whole digestion process.

Research has illustrated the instability of TPC in a simulated digestion (Mtolo, Gerrano, & Mellem, 2017). Both raw materials and pastes displayed increased TPC after the gastric phase, which subsequently declined after the intestinal phase, albeit above the undigested levels. Cebeci and Şahin-Yeşilçubuk (2014) reported that combinations with milk generally resulted in a decrease of TPC and TFC as well as inhibition of antioxidant activities when evaluating the matrix effect of blueberry, oatmeal and milk on their polyphenols and antioxidant activities after *in vitro* digestion. This discrepancy might be related to differences in the food matrix characteristics and the *in vitro* conditions of digestion. However, Sengul *et al.* (2014) observed a higher recovery of TPC after the gastric digestion of the fruit extract. This finding was possibly due to an increase in the flavylum cation in the acidic solution during the gastric phase of digestion, which is in agreement with results from this chapter. Therefore, it can be assumed that the increased values of TPC and TFC during the gastric phase are due to the acidic hydrolysis of phenolic glycosides to their aglycones. Furthermore, the decline in TFC values, subsequently resulted in the decreased TPC values in the intestinal phase. This is attributed to the degradation of phenolic compounds in the weak alkaline environment ($\text{pH} = 7.4$), particularly flavonoids, which are highly sensitive to alkaline conditions. According to a study from Fawole and Opara (2016), the decrease in phenolic compounds, notably anthocyanins in the intestinal phase of the *in vitro* digestion, was attributed to the transformation of the

flavylium cation to the colourless when the digestion medium became alkaline.

5.3.2 Changes in the anthocyanidin content in extracts during *in vitro* digestion

The anthocyanidin pigment experiences a reversible structural transformation with changes of the pH value, and these changes are reflected on the absorption spectrum. Thus the pH differential method could measure the values rapidly and accurately (Khoo *et al.*, 2017). **Table 5-3, 5-4, and Table 5-5** illustrate the changes in the anthocyanidin contents in the extracts of raw materials, blueberry and blackcurrant enriched pastes, respectively, during *in vitro* digestion. Cyanidin (Cyd), delphinidin (Dpd), malvidin (Mvd), peonidin (Pnd), and pelargonidin (Pg) were identified. Cyd accounted for the highest content ($p < 0.01$) among these five anthocyanidins, followed by Pg. Before the *in vitro* digestion, blackcurrant powder had the highest content of anthocyanidins ($p < 0.01$), followed by the extract of blueberry powder. Oat bran extract exhibited the lowest anthocyanidin content. As the level of the blueberry and blackcurrant powders added into the oat bran increased, the anthocyanidin content in the extract of blueberry and blackcurrant enriched pastes also increased. After the pasting procedure, the anthocyanidin content in pure oat bran paste extract could not be detected, as was the case in the intestinal digestion of oat bran and 10% blueberry enriched paste. Although the anthocyanin content decreased after undergoing *in vitro* digestion in all samples, the extent of this decrease differed from each other. Specifically, all anthocyanidins of the extract from blueberry powder that had undergone intestinal digestion decreased 99% compared with the extract of undigested blueberry powder, while the extract of 25% blueberry enriched paste decreased 80% for all anthocyanidins. The values of Dpd, Mvd, and Pg were higher in the extract of intestinal digested 25% blueberry enriched paste compared with the corresponding blueberry powder extract ($p < 0.01$). In contrast, blueberry powder extract had much higher Dpd, Mvd, and Pg before digestion than 25% blueberry enriched paste extract. These results may be due to the change in pH. As a food colourant, anthocyanin

with a lower pH (acidic conditions) has a significant effect, which gives a red pigment (Jamei & Babaloo, 2017; Ya Liu *et al.*, 2018; Song, Ji, Park, Kim, & Hogstrand, 2018). Each sample has its unique anthocyanidin profile as the anthocyanidin synthesis was strictly regulated by the genes of the corresponding enzymes involved in the respective biosynthetic pathways (Bu *et al.*, 2020). The results suggest that the pasting process, and enrichment with blueberry powder or blackcurrant powder for oat bran, caused the difference of anthocyanidin content. However, it did not change their main anthocyanidin profiles. Therefore, the extracts from pastes could be used to study the food matrix effects of the blueberry and blackcurrant enriched oat bran products.

Changes in the TPC, TFC, and the anthocyanidin content were influenced by the combined effects of dietary supplements and the phase of *in vitro* digestion and the effects were considered extremely significant ($p < 0.01$). Crucially, these results show that the changes in TPC in extracts from raw materials and pastes were not constant throughout the *in vitro* digestion procedure. According to the percentage of total variation, for TPC and TFC, digestion phase accounted for the most significant variation, indicating that the digestion process most likely contributes to the release of bioactive compounds due to the actions of the digestive enzymes' action, temperature and pH conditions (Bhatt & Patel, 2013). With respect to the anthocyanidin content, the different sources and proportions of supplements were the main reasons for the variation, revealing that the anthocyanins from different food sources are influenced by their food matrix, affecting on their chemical structures and stability as well as their bioavailability.

Table 5-1 TPC in extracts of raw materials and pastes (mg GAE/g sample)

Group	<i>In vitro</i> digestion phase			<i>Total variation</i>		
	Before digestion	Gastric	Intestinal	Samples (A)	Digestion phase (B)	A*B
Raw material				6.83% ($p < 0.001$)	86.10% ($p < 0.001$)	7.03% ($p < 0.001$)
Oat bran	1.49 ± 0.01^{Cc}	5.02 ± 0.02^{Ac}	2.26 ± 0.04^{Bc}			
Blueberry	84.91 ± 2.63^{Cb}	103.17 ± 1.15^{Ab}	91.23 ± 0.54^{Bb}			
Blackcurrant	97.15 ± 5.31^{Ca}	186.70 ± 0.23^{Aa}	122.78 ± 0.67^{Ba}			
Paste				28.50% ($p < 0.001$)	65.20% ($p < 0.001$)	6.14% ($p < 0.001$)
OBP	0.43 ± 0.01^{Cm}	4.26 ± 0.00^{Am}	2.25 ± 0.09^{Bn}			
ABB₁₀	0.53 ± 0.07^{Cm}	5.22 ± 0.14^{Al}	3.53 ± 0.05^{Bm}			
ABB₁₅	0.74 ± 0.06^{Cl}	5.24 ± 0.14^{Al}	3.92 ± 0.02^{Bl}			
ABB₂₅	1.60 ± 0.06^{Ck}	6.21 ± 0.23^{Ak}	4.98 ± 0.09^{Bk}			
ABC₁₀	2.93 ± 0.17^{Cj}	14.08 ± 0.65^{Aj}	7.52 ± 0.27^{Bj}			
ABC₁₅	5.85 ± 0.18^{Ci}	15.89 ± 0.40^{Ai}	10.44 ± 0.31^{Bi}			
ABC₂₅	10.82 ± 0.36^{Ch}	18.06 ± 0.19^{Ah}	11.96 ± 0.08^{Bh}			

Values are mean \pm standard deviation, $n = 3$. Raw materials and pastes were compared separately. Values with different uppercase letters, in the same row, are statistically different ($p < 0.05$), while values with different small case letters, in the same column, are statistically different ($p < 0.05$). TPC = total phenolic content; OBP = pure oat bran paste; ABB₁₀, ABB₁₅ and ABB₂₅ = oat bran paste enriched with 10%, 15% and 25% blueberry powder, respectively; ABC₁₀, ABC₁₅ and ABC₂₅ = oat bran paste enriched with 10%, 15% and 25% blackcurrant powder, respectively. All values are based on dry basis.

Table 5-2 TFC in extracts of raw materials and pastes (mg RE/g sample)

Group	<i>In vitro</i> digestion phase			Total variation		
	Before digestion	Gastric	Intestinal	Samples (A)	Digestion phase (B)	A*B
Raw material				18.20% (p < 0.001)	74.70% (p < 0.001)	6.80% (p < 0.001)
Oat bran	2.82 ± 0.42 ^{Bc}	4.77 ± 0.51 ^{Ac}	ND			
Blueberry	48.25 ± 2.31 ^{Ba}	65.48 ± 1.23 ^{Aa}	27.77 ± 2.39 ^{Ca}			
Blackcurrant	36.51 ± 0.93 ^{Bb}	43.32 ± 1.22 ^{Ab}	18.84 ± 1.40 ^{Cb}			
Paste				32.30% (p < 0.001)	52.50% (p < 0.001)	13.50% (p < 0.001)
OBP	0.96 ± 0.07 ^{Bm}	2.00 ± 0.19 ^{An}	ND			
ABB₁₀	5.82 ± 0.39 ^{Bk}	10.07 ± 0.47 ^{Al}	2.13 ± 0.09 ^{Cl}			
ABB₁₅	11.17 ± 0.90 ^{Bi}	21.27 ± 0.87 ^{Aj}	5.26 ± 0.06 ^{Cj}			
ABB₂₅	24.51 ± 6.15 ^{Bh}	37.62 ± 2.71 ^{Ah}	7.66 ± 0.19 ^{Ch}			
ABC₁₀	2.46 ± 0.10 ^{Bl}	7.95 ± 0.08 ^{Am}	1.55 ± 0.26 ^{Cm}			
ABC₁₅	7.29 ± 0.76 ^{Bj}	17.04 ± 0.44 ^{Ak}	3.10 ± 0.50 ^{Ck}			
ABC₂₅	16.89 ± 2.07 ^{Bh}	27.95 ± 0.61 ^{Ai}	6.94 ± 0.08 ^{Ci}			

Values are mean ± standard deviation, n = 3. Raw materials and pastes were compared separately. Values with different uppercase letters, in the same row, are statistically different ($p < 0.05$), while values with different small case letters, in the same column, are statistically different ($p < 0.05$). TFC = total flavonoid content. OBP = pure oat bran paste; ABB₁₀, ABB₁₅ and ABB₂₅ = oat bran paste enriched with 10%, 15% and 25% blueberry powder, respectively; ABC₁₀, ABC₁₅ and ABC₂₅ = oat bran paste enriched with 10%, 15% and 25% blackcurrant powder, respectively. All values are based on dry basis.

Table 5-3 Identification of anthocyanidin profiles of raw materials before and after *in vitro* digestion (mg/ 100 g sample)

	Oat bran extract			Blueberry powder extract			Blackcurrant powder extract		
	Before digestion	Gastric	Intestinal	Before digestion	Gastric	Intestinal	Before digestion	Gastric	Intestinal
Cyanidin	2.11 ± 0.02 ^{Ac}	0.26 ± 0.01 ^{Bc}	ND	949.20 ± 9.43 ^{Ab}	16.76 ± 0.23 ^{Bb}	9.61 ± 0.17 ^{Cb}	1677.00 ± 28.93 ^{Aa}	56.00 ± 4.49 ^{Ba}	45.01 ± 4.43 ^{Ca}
Delphinidin	1.75 ± 0.09 ^{Ac}	0.18 ± 0.01 ^{Bc}	ND	714.40 ± 8.69 ^{Ab}	13.00 ± 0.23 ^{Bb}	7.39 ± 0.15 ^{Cb}	1252.28 ± 45.50 ^{Aa}	41.34 ± 3.68 ^{Ba}	33.30 ± 4.22 ^{Ca}
Malvidin	1.74 ± 0.05 ^{Ac}	0.19 ± 0.01 ^{Bc}	ND	726.91 ± 7.63 ^{Ab}	13.27 ± 0.27 ^{Bb}	7.53 ± 0.15 ^{Cb}	1261.59 ± 26.51 ^{Aa}	41.66 ± 3.14 ^{Ba}	34.74 ± 5.21 ^{Ca}
Pelargonidin	2.62 ± 0.03 ^{Ac}	1.30 ± 0.01 ^{Bc}	ND	1204.20 ± 18.64 ^{Ab}	21.38 ± 0.67 ^{Bb}	12.12 ± 0.15 ^{Cb}	2148.34 ± 74.22 ^{Aa}	69.81 ± 3.30 ^{Ba}	56.98 ± 4.22 ^{Ca}
Peonidin	1.42 ± 0.03 ^{Ac}	0.17 ± 0.01 ^{Bc}	ND	656.82 ± 8.91 ^{Ab}	11.76 ± 0.42 ^{Bb}	6.65 ± 0.16 ^{Cb}	1163.49 ± 34.21 ^{Aa}	38.43 ± 4.14 ^{Ba}	30.42 ± 3.97 ^{Ca}

Values are mean ± standard deviation, n = 3. Each of anthocyanin was compared separately. The difference at the different digestion phase of each sample is expressed by uppercase letters, while the difference between different samples at the same digestion phase is expressed by small case letters ($p < 0.05$). All values are based on dry basis. ND = no data.

Table 5-4 Identification of anthocyanidin profiles of blueberry enriched paste extract before and after *in vitro* digestion (mg/ 100 g sample)

	10% blueberry enriched paste extract			15% blueberry enriched paste extract			25% blueberry enriched paste extract		
	Before digestion	Gastric	Intestinal	Before digestion	Gastric	Intestinal	Before digestion	Gastric	Intestinal
Cyanidin	44.25 ± 2.14 ^{Ac}	0.70 ± 0.04 ^{Bc}	ND	62.44 ± 0.59 ^{Ab}	3.19 ± 0.19 ^{Bb}	2.44 ± 0.36 ^{Cb}	77.96 ± 0.85 ^{Aa}	4.19 ± 0.17 ^{Ba}	1.16 ± 0.28 ^{Ca}
Delphinidin	34.61 ± 1.83 ^{Ac}	0.56 ± 0.04 ^{Bc}	ND	47.56 ± 0.38 ^{Ab}	2.39 ± 0.19 ^{Bb}	1.50 ± 0.17 ^{Cb}	59.91 ± 1.31 ^{Aa}	39.12 ± 0.49 ^{Ba}	10.78 ± 0.69 ^{Ca}
Malvidin	35.38 ± 1.97 ^{Ac}	0.54 ± 0.00 ^{Bc}	ND	48.67 ± 0.08 ^{Ab}	2.43 ± 0.20 ^{Bb}	1.45 ± 0.12 ^{Cb}	61.11 ± 21.39 ^{Aa}	39.99 ± 0.30 ^{Ba}	11.25 ± 0.30 ^{Ca}
Pelargonidin	55.61 ± 1.83 ^{Ac}	0.86 ± 0.02 ^{Bc}	ND	78.01 ± 0.56 ^{Ab}	4.02 ± 0.17 ^{Bb}	2.57 ± 0.33 ^{Cb}	97.17 ± 1.64 ^{Aa}	65.97 ± 0.64 ^{Ba}	15.97 ± 0.67 ^{Ca}
Peonidin	30.46 ± 1.87 ^{Ac}	0.48 ± 0.01 ^{Bc}	ND	43.03 ± 0.09 ^{Ab}	2.21 ± 0.21 ^{Bb}	1.48 ± 0.15 ^{Cb}	54.25 ± 1.22 ^{Aa}	36.05 ± 0.82 ^{Ba}	8.90 ± 0.40 ^{Ca}

Values are mean ± standard deviation, n =3. Each of anthocyanin was compared separately. The difference at the different digestion phase of each sample is expressed by uppercase letters, while the difference between different samples at the same digestion phase is expressed by small case letters ($p < 0.05$). All values are based on dry basis. ND = no data.

Table 5-5 Identification of anthocyanidin profiles of blackcurrant enriched paste extract before and after *in vitro* digestion (mg/ 100 g sample)

	10% blackcurrant enriched paste extract			15% blackcurrant enriched paste extract			25% blackcurrant enriched paste extract		
	Before digestion	Gastric	Intestinal	Before digestion	Gastric	Intestinal	Before digestion	Gastric	Intestinal
Cyanidin	122.65 ± 6.20 ^{Ac}	5.12 ± 0.11 ^{Bc}	3.82 ± 0.18 ^{Cb}	194.94 ± 9.08 ^{Ab}	11.26 ± 0.29 ^{Bb}	3.81 ± 0.17 ^{Cb}	394.42 ± 9.24 ^{Aa}	17.37 ± 0.55 ^{Ba}	9.94 ± 0.80 ^{Ca}
Delphinidin	91.20 ± 6.58 ^{Ac}	3.68 ± 0.08 ^{Bc}	2.74 ± 0.20 ^{Cb}	146.52 ± 7.88 ^{Ab}	8.40 ± 0.23 ^{Bb}	2.82 ± 0.12 ^{Cb}	293.44 ± 9.27 ^{Aa}	13.00 ± 0.46 ^{Ba}	7.75 ± 0.73 ^{Ca}
Malvidin	93.11 ± 6.63 ^{Ac}	3.75 ± 0.07 ^{Bc}	2.81 ± 0.18 ^{Cb}	150.91 ± 6.06 ^{Ab}	8.44 ± 0.40 ^{Bb}	2.87 ± 0.11 ^{Cb}	298.17 ± 8.37 ^{Aa}	13.41 ± 0.74 ^{Ba}	7.88 ± 0.74 ^{Ca}
Pelargonidin	146.88 ± 15.65 ^{Ac}	6.34 ± 0.12 ^{Bc}	4.78 ± 0.22 ^{Cb}	247.10 ± 7.13 ^{Ab}	14.03 ± 0.69 ^{Bb}	4.80 ± 0.16 ^{Cb}	500.11 ± 11.34 ^{Aa}	22.08 ± 0.94 ^{Ba}	12.57 ± 0.71 ^{Ca}
Peonidin	84.19 ± 6.58 ^{Ac}	3.45 ± 0.11 ^{Bc}	2.65 ± 0.20 ^{Cb}	136.61 ± 7.50 ^{Ab}	7.98 ± 0.27 ^{Bb}	2.62 ± 0.14 ^{Cb}	272.72 ± 9.58 ^{Aa}	12.06 ± 0.46 ^{Ba}	6.87 ± 0.90 ^{Ca}

Values are mean ± standard deviation, n = 3. Each of anthocyanin was compared separately. The difference at the different digestion phase of each sample is expressed by uppercase letters, while the difference between different samples at the same digestion phase is expressed by small case letters ($p < 0.05$). All values are based on dry basis. ND = no data.

5.3.3 Changes in antioxidant activities during *in vitro* digestion

The radical scavenging activity of the samples prior to digestion, reflected the same trend as the TPC levels in all samples, with blackcurrant > blueberry > blackcurrant enriched pastes > blueberry enriched pastes > oat bran > oat bran paste (**Fig. 5-1A & 5-1B**, respectively). After the gastric phase, the radical scavenging activity of both raw materials and pastes decreased, varying from a 13% to 33% reduction in DPPH assay and from a 20% to 89% reduction in the ABTS assay ($p < 0.01$). The radical scavenging activity of the raw materials after the intestinal phase was lower than that of the corresponding undigested extracts. Both blueberry and blackcurrant enriched pastes seemed to be effective in scavenging the DPPH and ABTS^{•+} free radical, and contrary to the raw materials, the radical scavenging activity of these pastes were higher after the intestinal digestion compared to the radical scavenging activity exhibited by the undigested paste ($p < 0.01$). The DPPH values of the intestinal digesta of blueberry and blackcurrant enriched pastes increased between 1- and 1.54-fold compared with the undigested paste ($p < 0.01$). The same trend was observed for the ABTS assay results, with the scavenging values of the intestinally digested blueberry and blackcurrant enriched pastes being higher by 14 to 53%, compared to the corresponding undigested paste ($p < 0.01$). The reducing antioxidant powers measured by FRAP of the raw materials and pastes were consistent with the TPC measured in the undigested paste (**Fig. 5-1C**). Overall, blackcurrant powder showed the highest antioxidant power (714 mmol FeSO₄/g), followed by blueberry powder (282 mmol FeSO₄/g). In terms of the pastes, oat bran paste showed the lowest antioxidant power (13 mmol FeSO₄/g). With increasing levels of enrichment, the FRAP value of blueberry and blackcurrant enriched pastes increased, ranging between 0.9- and 6.2-fold compared with the oat bran paste ($p < 0.01$). Blackcurrant enriched paste showed a stronger reducing power than blueberry enriched paste. Interestingly, the FRAP values increased significantly at the gastric phase for all samples, perhaps as a result of the TPC at this phase.

However, the reducing powers then generally decreased ($p < 0.01$) by 7% to 76% at the intestinal phase, with larger decreases observed in the raw materials values compared with those observed for the pastes. Although there was a decrease after the final phase, FRAP values after the intestinal digestion remained higher than the undigested extracts ($p < 0.01$). The fluctuation of the reducing power, in all of the samples, could be due to the pH of the medium. The pH of a substance is known to influence molecule racemisation, possibly creating two chiral enantiomers with different reactivity. Therefore, some antioxidants could be more sensitive at acidic pH at the gastric phase and less reactive at alkaline pH at the intestinal phase. Bouayed, Hoffmann, and Bohn (2011) reported a similar trend of reducing powder in apple varieties. They found that the reducing antioxidant capacity present in apple varieties, as determined in methanolic extracts, was significantly higher compared to those found in gastric digesta for all apple varieties, while the reducing power present in the intestinal digesta of apple varieties was lower than those found in gastric digesta. Regarding the change in reduction of antioxidant power from the gastric phase to the intestinal phase, the phenolics responsible for ferric reduction may reduce or convert to certain metabolites with different chemical properties, as these polyphenols are highly sensitive to alkaline conditions (Del Rio *et al.*, 2010).

Çelik, Gökmen, and Fogliano (2013) reported that once cereal products are consumed, the large proportion of insoluble antioxidants bound to dietary fibres reach the colon without digestion and become bound antioxidant radicals themselves, while soluble antioxidants regenerate bound antioxidant radicals, thereby prolonging their antioxidant action longer. Masisi, Beta, and Moghadasian (2016) also reported that after the consumption of breakfast cereals, antioxidant activities increased significantly after the *in vitro* digestion compared to the chemical solvent extraction procedure used for undigested samples. These reports explain the increased reducing power of all blueberry and blackcurrant enriched pastes. Antioxidant

activities of foods varies depending on the content of phenolic compounds, flavonoids, protein, lipid and carbohydrates (Oniszczuk *et al.*, 2019).

5.3.4 Pearson's correlations between phenolic compounds and antioxidant assays

The correlation coefficients between TPC, TFC, and the anthocyanidin content (**Table 5-6**) and antioxidant activity values, by three assays, were recorded before, and after the *in vitro* digestion. Strong positive correlations were observed between these phenolic compounds and antioxidant activity values. The anthocyanidin content had stronger correlations with antioxidant activity values compared to TPC and TFC. As shown in **Table 5-6**, the correlation coefficients between the anthocyanidin content and DPPH values were all larger than 0.97 in undigested ($R^2 = 0.992, p < 0.01$), gastric digested ($R^2 = 0.982, p < 0.01$), and digested samples ($R^2 = 0.979, p < 0.01$), followed by the FRAP values, which had the stronger correlations in undigested and gastric digested samples ($R^2 = 0.996, p < 0.01$) when compared to the intestinal digested samples. These strong correlations revealed that the phenolic compounds from blueberry and blackcurrant were highly responsible for their antioxidant activity throughout the whole digestion process. In chapter 4, the nutritional components in the raw materials and pastes, and the negative correlations were observed between antioxidant activity (DPPH, ABTS⁺ and FRAP) and nutritional components, including fat, protein and total starch content, while strong positive correlations were found between antioxidant activity and p-coumaric acid content and gallic acid content. Previously metabolites formed as a result of structural changes brought about by the alkaline conditions have been shown to have a different reactivity in the FRAP assay (Gulcin, 2020). However, some insoluble antioxidant compounds remaining in indigestible materials may be underestimated by the three assays (Cömert & Gökmen, 2017).

5.4 Conclusion

In this chapter, oat bran enriched with 10%, 15%, and 25% blackcurrant powder or blueberry powder increased the content of bioactive ingredients compared to the pure oat bran paste, in particular of anthocyanidins, including cyanidin, delphinidin, malvidin, pelargonidin, and peonidin. The TPC and the antioxidant capacity estimated by DPPH, ABTS, and FRAP assays of raw materials and pastes increased, while the TFC and five major anthocyanidins contents decreased after the *in vitro* digestion. Such observations demonstrated the potential for enhancing the TPC as well as the *in vitro* antioxidant capacity of the blueberry and blackcurrant enriched oat bran products, which need to be investigated further for the development of food and nutraceutical health promoting supplements. Furthermore, for the future work, the combination of the cell line with the *in vivo* study on mice should be conducted and the study would focus on the production of the metabolites of polyphenols that are predominant in the circulation.

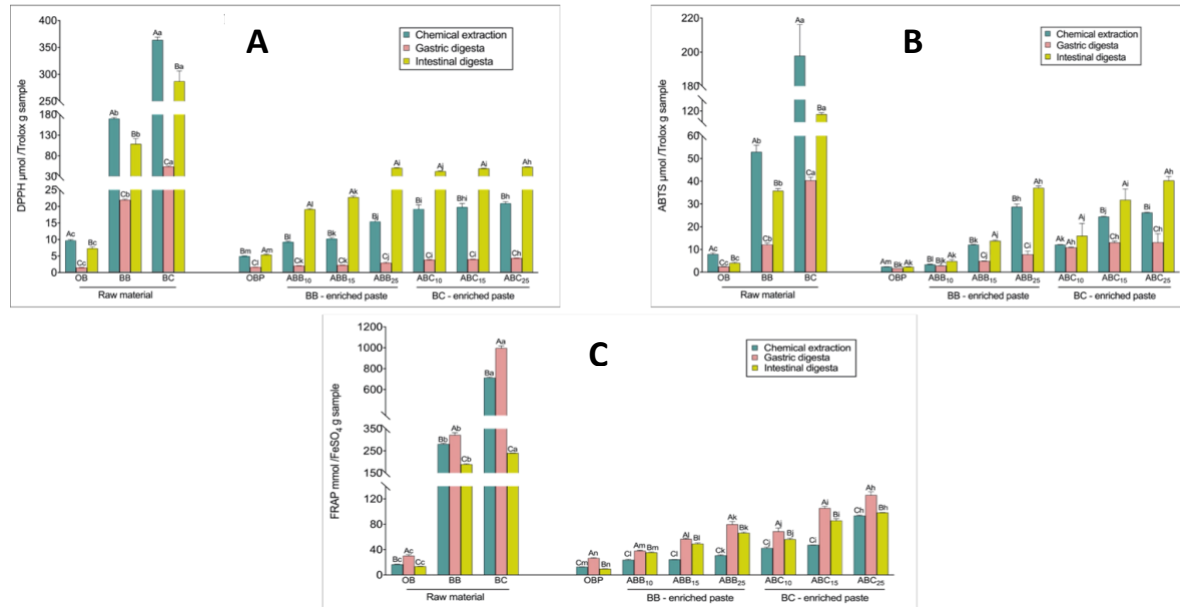


Figure 5.1 Changes in antioxidant activities during *in vitro* digestion

DPPH values (A), ABTS values (B), and FRAP values (C), respectively. Raw materials and pastes were compared separately. Values with different uppercase letters at different digestion phase in one sample are statistically different, while values of bars in the same colour with different small case letters are statistically different ($p < 0.05$). OBP = pure oat bran paste; ABB₁₀, ABB₁₅ and ABB₂₅ = oat bran paste enriched with 10%, 15% and 25% blueberry powder, respectively; ABC₁₀, ABC₁₅ and ABC₂₅ = oat bran paste enriched with 10%, 15% and 25% blackcurrant powder, respectively.

Table 5-6 Pearson's correlation between phenolic compounds and three antioxidant assays values

	Before digestion	Gastric	Intestinal
Pearson's correlation between TPC and three antioxidant assays values			
DPPH values	0.8708 ^{**}	0.9783 ^{**}	0.8641 ^{**}
ABTS values	0.6867 ^{**}	0.7748 ^{**}	0.6941 ^{**}
FRAP values	0.8449 ^{**}	0.9382 ^{**}	0.9279 ^{**}
Pearson's correlation between TFC and three antioxidant assays values			
DPPH values	0.5724 [*]	0.3737	0.4996 [*]
ABTS values	0.4333 [*]	0.2707	0.4245 [*]
FRAP values	0.5080 [*]	0.3279	0.7968 ^{**}
Pearson's correlation between the anthocyanin content and three antioxidant assays values			
DPPH values	0.9922 ^{**}	0.9820 ^{**}	0.9790 ^{**}
ABTS values	0.9551 ^{**}	0.9650 ^{**}	0.9188 ^{**}
FRAP values	0.9951 ^{**}	0.9962 ^{**}	0.8376 ^{**}

^{*} $p < 0.05$, ^{**} $p < 0.01$, $n = 3$. TPC = total phenolic content; TFC = total flavonoid content.

Chapter 6

Evaluation of the extracts from blueberry and blackcurrant powder enriched oat bran paste on the *in vitro* inhibitory activity of α -amylase

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Abstract

The α -amylase inhibitory activities of the extracts from raw materials (oat bran, blueberry and blackcurrant powders), and oat bran paste enriched with 25% blueberry powder or blackcurrant powder, respectively, were studied by measuring their half inhibitory (IC₅₀) concentrations. Addition of blueberry or blackcurrant powder into oat bran paste increased the α -amylase inhibitory activity with decreased IC₅₀ values. The content of main anthocyanins was measured by the pH differential method, and the potential inhibitory mechanisms of these extracts were also investigated by detailed inhibition kinetics and molecular docking study. The results showed that cyanidin and delphinidin were the main anthocyanidin profiles in extracts. The extract of blueberry powder showed the strongest α -amylase inhibitory activity with its IC₅₀ of 0.73 mg/mL. Among the extracts of pastes, the extract of 25% blueberry enriched paste exhibited the strongest α -amylase inhibitory activity (IC₅₀ = 5.71 mg/mL). Only blackcurrant powder extract was a competitive inhibitor, while other extracts were all mixed-type inhibitors against α -amylase. The docking studies revealed that the α -amylase inhibitory activity by extracts was potentially driven by hydrogen bonding, cyanidin-3-glucoside and delphinidin-3-glucoside had stronger binding affinity compared to malvidin-3-glucoside and cyanidin-3-rutinside. This chapter suggested that supplementary of

blueberry and blackcurrant with oat bran could be a potential source of bioactive products for anti-diabetic activity.

Keywords: anthocyanins, α -amylase, inhibition kinetics, molecular docking; anti-diabetic

6.1 Introduction

Type II diabetes (T2DM) is defined as a chronic disease due to the reduced insulin sensitivity, which can lead to multiple complications. Regulation of postprandial hyperglycaemia is recommended for the prevention and treatment of T2DM (Ormazabal *et al.*, 2018). Controlling starch breakdown and retarding the intestinal glucose absorption by inhibiting digestive enzymes, such as α -amylase which plays an important role in the digestion of starch, are strategies which are considered as a potential therapeutic approach (Dhital, Gidley, & Warren, 2015). Alpha-amylase is an enzyme that hydrolyses α -1,4-glucan polysaccharides, such as starch and glycogen, producing glucose oligosaccharides prior to glucose adsorption, which triggers an insulin response when glucose adsorption occurs too quickly (Röder *et al.*, 2016). Alpha-amylase is the main form of amylase found in mammals (Mahmood, 2016). It is also present in plant seeds containing starch as a food reserve, and is excreted by some fungi (Mehta & Satyanarayana, 2016). As α -amylase is a calcium metalloenzyme, and the calcium and chloride ions are presented at the active site of the enzyme, which are essential for the maintenance of the tertiary structure and catalytic activities of α -amylase (Franco, Rigden, Melo, & Grossi-de-Sá, 2002). In addition, some aromatic residues, such as, Tyr62, Trp59, and Trp58, are stacking features at the entrance of the active site of the enzyme (Larson, Day, & McPherson, 2010). In particular, it is proposed that the residues of amino acids at the active site of α -amylase should be in positions, where the interactions between the inhibitors and α -amylase are thought to occur (Luo *et al.*, 2020; Sun, Wang, & Miao, 2020). Even though these digestive enzyme inhibitors, such as acarbose, are commonly used to regulate the

postprandial hyperglycaemia in T2DM, these inhibitors may have severe side effects, such as hepatotoxicity (Agu *et al.*, 2019; Lewis & Kleiner, 2012).

The screening of natural inhibitors for carbohydrate digestive enzymes has been viewed as alternative prevention and treatment of T2DM due to their low toxicity (Chipiti, Ibrahim, Singh, & Islam, 2015; Lankatillake, Huynh, & Dias, 2019). Some plant extracts have been documented to have bioactivity that can support human health through the inhibition of carbohydrate hydrolysing enzymes (Duarte, Guarino, Barroso, & Gil, 2020; Wang, Wang, & Chan, 2013), and flavonoids with several hydroxyl groups are the main active ingredients in these plant extracts. Hydroxyl groups are important for the inhibitory action of flavonoid compounds towards α -amylase since this inhibitory activity seems to rely on the formation of hydrogen bonds among the -OH groups of phenolics and the side chains of amino acids at the active sites of α -amylase (Franco *et al.*, 2020). Flavonoids without the substitution of -CH₃ and -OCH₃ at -OH in their structures are more effective in inhibiting α -amylase compared to those with other certain substitution patterns (Asghari, Mafakheri, Zengin, Dinparast, & Bahadori, 2020). In addition, it has been shown that the methylation of hydroxyl groups in flavonoids minimise the inhibitory activity of α -amylase. These reports further confirm the key role of hydroxyl group of flavonoids in inhibiting the α -amylase activity (Lim, Zhang, Ferruzzi, & Hamaker, 2019; Proença *et al.*, 2019). Anthocyanin is a major subclass of flavonoids, which could be used to defend digestive enzymes from effective inhibitors (Kozłowska & Szostak-Węgierek, 2019).

It has been reported that berries could improve hypoglycaemic response by inhibiting the activities of α -amylase (Mcdougall & Stewart, 2005). Various coloured berries, especially blueberry and blackcurrant, are widely distributed in New Zealand (Braakhuis, Somerville, & Hurst, 2020; Buck *et al.*, 2012). The phenolic compounds in blueberry and blackcurrant have been shown to inhibit α -amylase (Grussu, Stewart, & McDougall, 2011; Ma *et al.*, 2018). Oat

bran has also been demonstrated to have positive effects on postprandial glucose levels and body mass improvement (Ulmius *et al.*, 2011).

Chapter 4 showed that 10%, 15%, and 25% of blueberry and blackcurrant enriched oat bran paste decreased the extent the starch digestion and improved the *in vitro* glycaemic response by decreasing the reducing sugar released over a 120-min *in vitro* digestion. In this chapter, the main anthocyanins in the extracts of blueberry and blackcurrant enriched pastes were identified by the pH differential method, and these were used to detect enzyme inhibitory activities. A 25% enrichment with blueberry and blackcurrant powder for oat bran was chosen to be included into the food matrix, and the effects on the inhibitory activities of α -amylase were determined by a combination of detailed inhibition kinetics and molecular docking.

6.2 Methods

6.2.1 Preparation of the oat bran paste

Pastes were prepared as described in 3.2.

6.2.2 Extraction of the raw materials and pastes

The extraction of samples was performed as outlined in 3.5.

6.2.3 Identification of the major anthocyanin content in extracts

The anthocyanidin profiles in extracts were identified as outlined in 3.11.

6.2.4 Alpha-amylase inhibitory activity and α -amylase inhibitory kinetics of extracts

The α -amylase inhibitory activity was performed followed by the method in 3.14.1, while the kinetics of α -amylase inhibition by extracts were determined as described in 3.14.2.

6.2.5 Molecular docking study on α -amylase

The molecular docking study on α -amylase was employed as outlined in 3.15.

6.2.6 Statistical analysis

Statistical analysis was carried out as outlined in 3.17.

6.3 Results and discussion

6.3.1 The content of major anthocyanins in extracts of raw materials and pastes

The content of individual cyanidins with the glycosides were investigated in the extracts of raw materials and pastes by the pH differential method, and the results are presented in **Table 6-1 & 6-2**, respectively. Cyanidin, delphinidin, malvidin, peonidin, petunidin, and pelargonidin were identified. Cyanidin and delphinidin were the major anthocyanins in the extracts of raw materials and pastes. Among these anthocyanins, Cya-3-Rut accounted for the highest content ($p < 0.01$), apart from oat bran paste extract in which peonidin 3-glucosidase was taken up the highest content. The total content of identified cyanidins with its glycosides decreased in the following order: blackcurrant powder (965206.6 $\mu\text{g}/100\text{ g}$) > blueberry powder (415722.6 $\mu\text{g}/100\text{ g}$) > oat bran (574.1 $\mu\text{g}/100\text{ g}$). In terms of the extracts of pastes, extracts from blueberry and blackcurrant enriched pastes had much higher anthocyanin contents compared to oat bran paste extract ($p < 0.05$). Cyanidins and delphinidin were the main anthocyanins in the extracts of pastes. The extract of the oat bran paste enriched with 25% blackcurrant powder had the highest anthocyanin content (25053 $\mu\text{g}/100\text{ g}$) ($p < 0.01$), followed by the extract from 25% blueberry powder enriched paste (14214 $\mu\text{g}/100\text{ g}$). By contrast, most of the anthocyanins could not be detected in the extract of oat bran paste, and this extract had the lowest total anthocyanin content, only 58.04 $\mu\text{g}/100\text{ g}$.

Table 6-1 The anthocyanin content in the extracts of raw materials (µg/ 100 g sample)

Anthocyanins	Oat bran	Blueberry powder	Blackcurrant powder
Cyanidin 3-glucoside	32.50 ± 8.17 ^C	14964.98 ± 1479.61 ^B	36266.40 ± 1685.65 ^A
Cyanidin 3-galactoside	29.43 ± 2.68 ^C	22331.22 ± 1093.50 ^B	50969.76 ± 758.64 ^A
Cyanidin 3-arabinoside	22.30 ± 5.80 ^C	12785.94 ± 1022.13 ^B	30766.48 ± 1124.33 ^A
Cyanidin 3-rutinoside	118.95 ± 1.57 ^C	96852.80 ± 71381.73 ^B	330616.73 ± 4189.10 ^A
Cyanidin 3,5-diglucoside	36.67 ± 2.57 ^C	25981.83 ± 965.68 ^A	63642.05 ± 288.04 ^B
Delphinidin 3-glucoside	41.78 ± 5.90 ^C	32524.68 ± 936.09 ^B	76135.00 ± 508.66 ^A
Delphinidin 3,5-diglucoside	34.63 ± 3.12 ^C	23896.39 ± 940.33 ^B	55447.28 ± 1348.64 ^A
Malvidin 3-glucoside	23.41 ± 1.34 ^C	18364.98 ± 483.70 ^B	43656.61 ± 1064.02 ^A
Malvidin 3-galactoside	19.53 ± 2.20 ^C	15010.95 ± 1293.31 ^B	36629.90 ± 332.21 ^A
Malvidin 3,5-diglucoside	19.81 ± 1.84 ^C	14458.41 ± 484.21 ^B	33650.44 ± 1761.28 ^A
Peonidin 3-glucoside	73.46 ± 5.55 ^C	53710.29 ± 1676.30 ^A	13170.93 ± 562.38 ^B
Petunidin 3-glucoside	63.16 ± 9.01 ^C	39121.91 ± 1223.33 ^B	91318.68 ± 1524.90 ^A
Pelargonidin 3-glucoside	58.51 ± 7.33 ^C	45718.20 ± 2283.03 ^B	102936.30 ± 741.32 ^A

Values are mean ± standard deviation. Values in the same row with different letters are statistically different ($p < 0.05$).

Table 6-2 The anthocyanin content in the extracts of pastes (µg/ 100 g sample)

Anthocyanins	Oat bran paste	ABB ₁₀	ABB ₁₅	ABB ₂₅	ABC ₁₀	ABC ₁₅	ABC ₂₅
Cyanidin 3-glucoside	ND	252.83 ± 23.37 ^d	301.30 ± 6.20 ^c	408.83 ± 3.45 ^b	402.10 ± 17.38 ^b	644.33 ± 2.78 ^a	661.90 ± 25.07 ^a
Cyanidin 3-galactoside	ND	363.90 ± 58.17 ^d	518.20 ± 57.66 ^c	722.37 ± 94.69 ^b	778.97 ± 43.80 ^b	1125.00 ± 55.25 ^a	1127.67 ± 39.27 ^a
Cyanidin 3-arabinoside	ND	227.37 ± 78.35 ^c	301.47 ± 53.21 ^c	446.97 ± 35.81 ^b	433.10 ± 50.97 ^b	649.97 ± 68.81 ^a	651.70 ± 62.70 ^a
Cyanidin 3-rutinoside	25.02 ± 0.55 ^g	2340.33 ± 158.18 ^f	2958.33 ± 82.97 ^e	4185.00 ± 200.91 ^d	4876.00 ± 163.68 ^c	7499.67 ± 10.69 ^b	7924.00 ± 207.56 ^a
Cyanidin 3,5-diglucoside	ND	468.10 ± 9.06 ^d	587.20 ± 10.54 ^c	857.73 ± 24.12 ^b	903.30 ± 36.86 ^b	1455.00 ± 21.66 ^a	1393.33 ± 129.41 ^a
Delphinidin 3-glucoside	6.07 ± 0.20 ^g	529.27 ± 42.72 ^f	760.37 ± 45.44 ^e	1053.67 ± 34.12 ^d	1247.33 ± 103.98 ^c	1728.33 ± 20.03 ^b	1832.33 ± 51.29 ^a
Delphinidin 3,5-diglucoside	ND	377.60 ± 41.42 ^c	617.17 ± 173.38 ^{bc}	856.30 ± 108.24 ^b	902.80 ± 47.69 ^b	1264.27 ± 113.51 ^a	1356.33 ± 35.23 ^a
Malvidin 3-glucoside	ND	333.77 ± 38.72 ^c	450.90 ± 113.12 ^{bc}	560.87 ± 64.42 ^b	608.73 ± 54.62 ^b	969.80 ± 46.42 ^a	953.00 ± 28.56 ^a
Malvidin 3-galactoside	ND	265.30 ± 68.84 ^c	354.97 ± 50.71 ^c	543.83 ± 58.48 ^b	539.90 ± 67.59 ^b	856.13 ± 49.59 ^a	912.10 ± 84.97 ^a
Malvidin 3,5-diglucoside	ND	242.20 ± 67.13 ^c	337.40 ± 67.41 ^c	518.43 ± 82.50 ^b	520.80 ± 35.55 ^b	801.57 ± 66.93 ^a	826.23 ± 85.39 ^a
Peonidin 3-glucoside	13.20 ± 1.10 ^e	996.03 ± 106.03 ^d	1272.00 ± 120.93 ^c	1631.33 ± 177.92 ^b	1820.67 ± 164.07 ^b	3006.33 ± 34.03 ^a	3177.33 ± 161.14 ^a
Petunidin 3-glucoside	10.21 ± 1.34 ^e	625.13 ± 54.21 ^d	863.97 ± 65.44 ^c	1088.80 ± 96.53 ^b	1150.00 ± 36.59 ^b	1710.00 ± 119.03 ^a	1877.00 ± 86.64 ^a
Pelargonidin 3-glucoside	3.57 ± 0.41 ^f	757.43 ± 97.79 ^e	1010.57 ± 116.42 ^d	1340.00 ± 51.96 ^c	1563.67 ± 2.08 ^b	2203.67 ± 168.65 ^a	2360.67 ± 172.94 ^a

Values are mean ± standard deviation, n = 3. Values with different letters in the same row are statistically different ($p < 0.05$). ABB₁₀, ABB₁₅ and ABB₂₅ = oat bran paste enriched with 10%, 15% and 25% blueberry powder, respectively; ABC₁₀, ABC₁₅ and ABC₂₅ = oat bran paste enriched with 10%, 15% and 25% blackcurrant powder, respectively. ND = no data.

6.3.2 Alpha-amylase inhibitory activity

Alpha-amylase is one of the major enzymes involved in the digestion of starchy food, releasing oligosaccharides that can be further degraded to glucose, and which are rapidly absorbed by the body. Consequently, inhibition of α -amylase activity is considered to be an effective strategy for managing diabetes (Dhital, Warren, Butterworth, Ellis, & Gidley, 2017). The previous chapters have illustrated that the major anthocyanidins profiles did not change with increasing the amount of the blueberry powder and blackcurrant powder into oat bran. Therefore, the extracts of the oat bran paste enriched with 25% blueberry powder and 25% blackcurrant powder were selected to study the effects of bioactive compounds from blueberry powder and blackcurrant powder on the inhibitory activity of oat bran on enzymes. **Fig. 6-1D** shows that the extracts of blueberry powder (among the extracts from raw materials) and 25% blueberry enriched paste (among the extracts from pastes) exhibited the strongest inhibitory activities for α -amylase, with IC_{50} of 0.73 mg/mL and 5.17 mg/mL, respectively ($p < 0.01$). However, they were still much lower when compared to the positive drug of acarbose ($IC_{50} = 0.06$ mg/mL) ($p < 0.01$). It is not surprising since acarbose is a purified synthetic α -amylase inhibitor (Tysoe *et al.*, 2016), whereas the α -amylase inhibitory activity in the extracts of the raw materials and pastes used in this study could be influenced by the complex food matrix. **Fig. 6-1A** (acarbose), **6-1B** (extracts of raw materials), and **6-1C** (extracts of pastes) show the α -amylase inhibition curves at multiple concentrations of inhibitors. The activity of α -amylase decreased with increasing concentrations of these inhibitors. Compared to the extract of blackcurrant powder, blueberry powder extract showed higher inhibitory activity ($p < 0.01$), while oat bran extract presented the lowest inhibitory effect with an IC_{50} of 12.35 mg/mL ($p < 0.01$). The extracts of 25% blueberry and 25% blackcurrant enriched paste exerted stronger α -amylase inhibitory activities compared to the extract of oat bran paste ($p < 0.01$). Notably, the inhibitory effects of all the inhibitors on α -amylase were in the following

order: Acarbose > blueberry powder extract > blackcurrant powder extract > 25% blueberry enriched paste extract > 25% blackcurrant enriched paste extract > oat bran extract > pure oat bran paste extract.

6.3.3 The α -amylase inhibitory kinetics of inhibitors

The Lineweaver-Burk equation, a double-reciprocal plot of Michaelis-Menten equation, was employed to study the detailed kinetic characterisation of the α -amylase inhibitory activity, V_{\max} and K_m (Robinson, 2015). However, in this plot, a fairly large error may occur when the concentration of substrates is very low (Kaeswurm, Claasen, Fischer, & Buchweitz, 2019). Additionally, the Lineweaver-Burk plot cannot distinguish between the different forms of inhibition, such as uncompetitive, non-competitive and mixed-type (Palmer & Bonner, 2011). Therefore, the Dixon and Hanes-Woolf plots were also used in this study to assist in the analysis of inhibition kinetics and further determination of the inhibition types. The enzyme velocity (v) in the absence and presence of inhibitor was determined by the analysis of Michaelis-Menten curve in **Fig. 6-2**. The Michaelis-Menten curve showed that under the same starch concentration, v decreased with increasing concentration of inhibitors, illustrating that the α -amylase inhibitory effect of the extracts from the raw materials and pastes was in an inhibitor concentration dependent manner. The extracts of blueberry and blackcurrant powders showed higher enzyme inhibitory activity compared to the oat bran extract ($p < 0.01$), while the extracts from 25% berry enriched pastes exhibited stronger enzyme inhibitory effects compared to the extract of pure oat bran paste ($p < 0.01$).

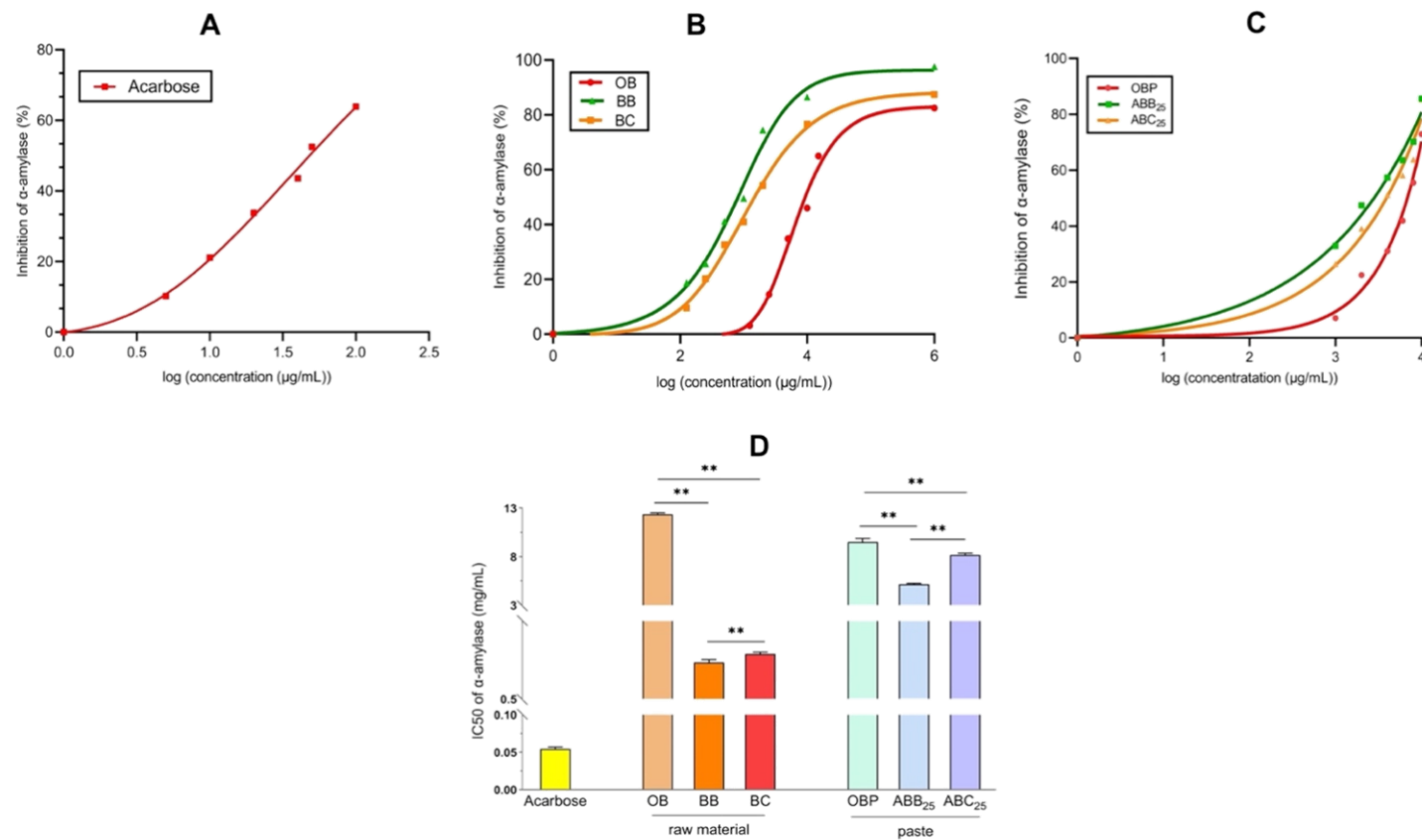


Figure 6.1 Inhibition curves and IC₅₀ of extracts against α -amylase

The inhibition α -amylase curves by inhibitors of acarbose (A), the extracts of raw materials (B) and pastes (C) with different concentrations; IC₅₀ of inhibitors against α -amylase (D). OB = oat bran; BB = blueberry powder; BC = blackcurrant powder; OBP = oat bran paste; ABB₂₅ = 25% blueberry enriched oat bran paste; ABC₂₅ = 25% blackcurrant enriched oat bran paste.

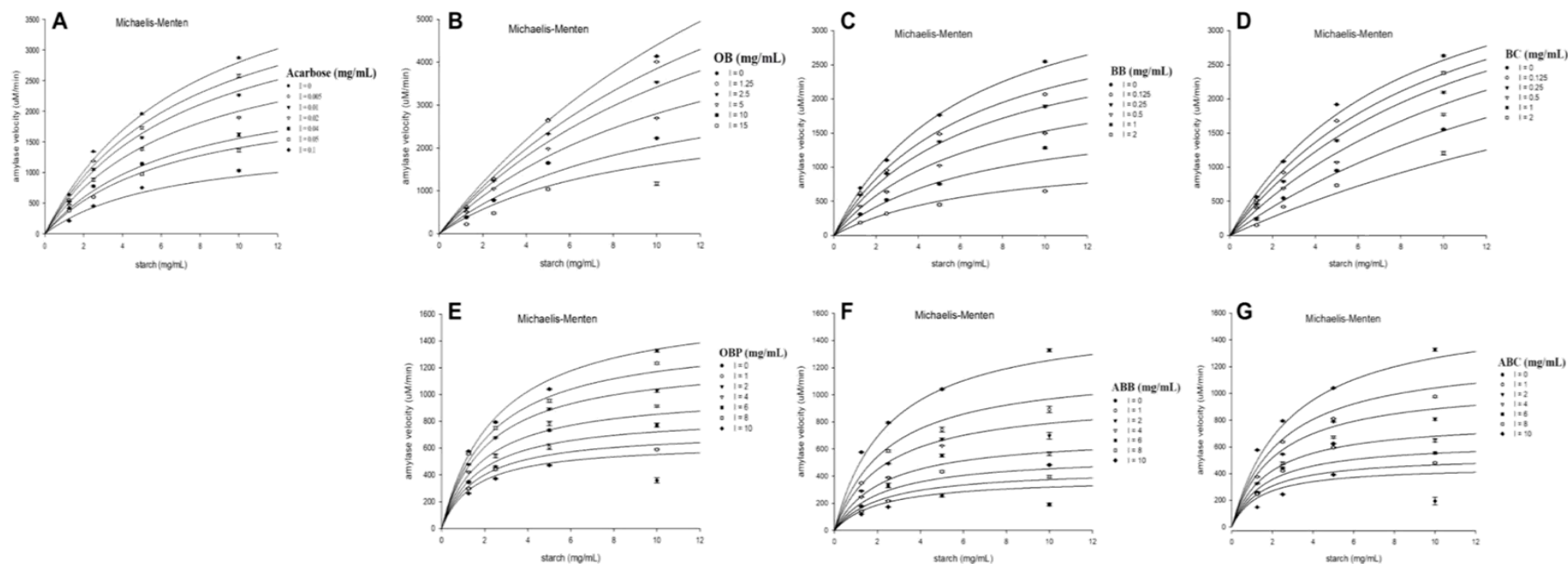


Figure 6.2 Michaelis-Menten for α -amylase inhibition by inhibitors

Inhibitors have illustrated in figures. Various concentrations of inhibitors are list in the legend entries. OB = oat bran; BB = blueberry powder; BC = blackcurrant powder; OBP = oat bran paste; ABB = 25% blueberry enriched oat bran paste; ABC = 25% blackcurrant enriched oat bran paste.

6.3.4 The Dixon & Hanes-Woolf plots for the α -amylase inhibitory activity

Information regarding the type of inhibition can be obtained from the combined use of Dixon and Hanes-Woolf plots. The Dixon plot of the blackcurrant powder extract shows a clear intersection, while its Hanes-Woolf plot presents lines running parallel with each other (**Fig. 6-4C**), illustrating that the mode of action of the extract from blackcurrant powder was through the competitive inhibition against α -amylase, which also revealed that the extract of blackcurrant powder exerted an inhibitory effect via binding to the active site of α -amylase (Wulan, Utomo, & Mahdi, 2015). Both the Dixon and the Hanes-Woolf plots of the extracts from oat bran (**Fig. 6-4B**), blueberry powder (**Fig. 6-4C**), and pastes (**Fig. 6-5**) showed that they intersected at one point, suggesting their mode of action on the α -amylase was through mixed inhibition, which includes competitive and uncompetitive inhibition. Therefore, the increased starch concentration could not completely alleviate the α -amylase inhibition by these inhibitors due to the presence of uncompetitive inhibition (Koh, Lu, & Zhou, 2020). Specifically, these inhibitors were able to compete with the starch solution not only in binding with the α -amylase, but also in binding with the α -amylase-starch complex, forming an 'inhibitor- α -amylase-starch' ternary complex.

K_{ic} and K_{iu} represent the dissociation constants of the enzyme-inhibitor and 'inhibitor-enzyme-substrate' complexes, respectively (Yoshino & Murakami, 2015). Therefore, a higher $1/K_{ic}$ and a lower $1/K_{iu}$ suggest the inhibitor is more likely to bind to the enzyme instead of the enzyme-substrate complex. The $1/K_{ic}$ value of the oat bran extract, oat bran paste extract, and the extracts from 25% blueberry and 25% blackcurrant enriched paste was lower than that of $1/K_{iu}$ value (**Table 6-3**), indicating that they could bind more tightly with the α -amylase-starch complex to exert their inhibitory effects. However, the $1/K_{ic}$ value of the extract from blueberry powder was slightly higher than the corresponding $1/K_{iu}$ value, indicating that the extract from blueberry powder could bind with the active site of α -amylase than non-active

site. It can explain the strong correlation between IC₅₀ and both K_{ic} ($R^2 = 0.932$, $p < 0.001$) (Fig. 6-6A), and K_{iu} ($R^2 = 0.852$, $p = 0.003$) (Fig. 6-6B). The extract of 25% blueberry enriched paste had a stronger α -amylase inhibitory effect than the extract of 25% blackcurrant enriched paste, while the extract of oat bran paste had the lowest effect on the α -amylase inhibition activity ($p < 0.01$). According to the anthocyanin content analysis, the α -amylase inhibitory effect may not only be contributed by the anthocyanin content

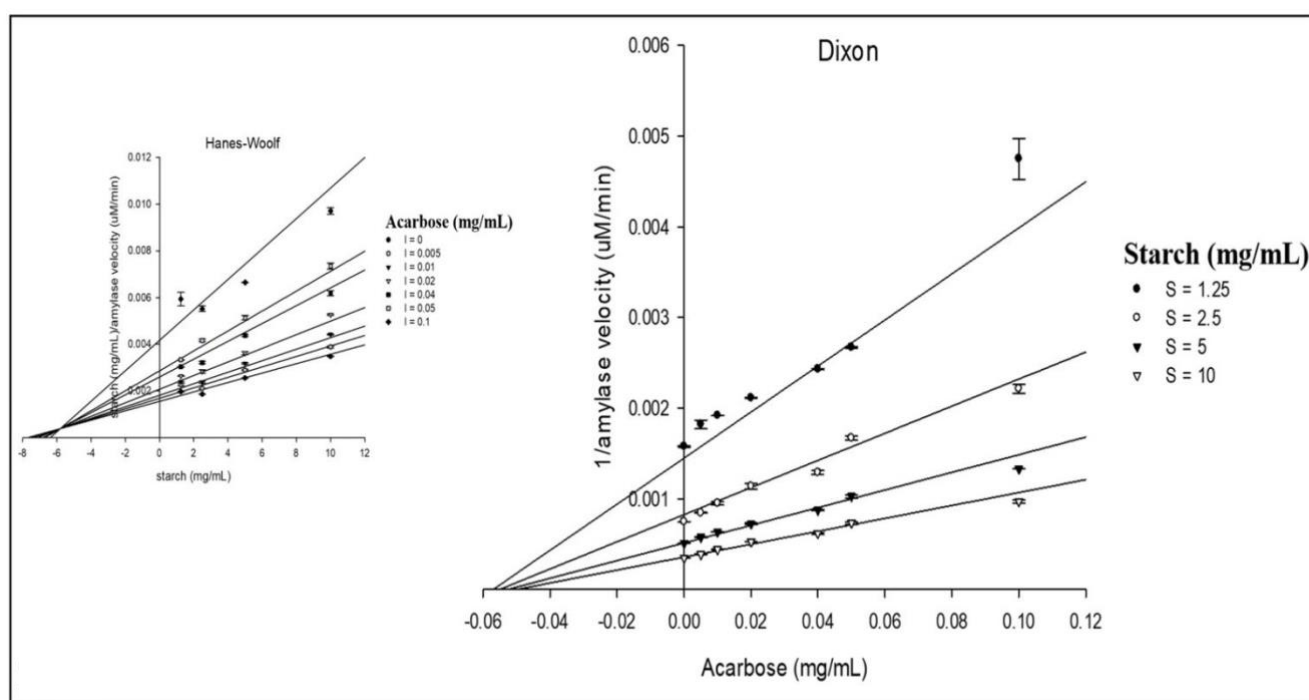


Figure 6.3 Dixon and Hanes-Woolf plot (insets) for α -amylase inhibition by acarbose

Various concentrations of acarbose are list in the legend entries.

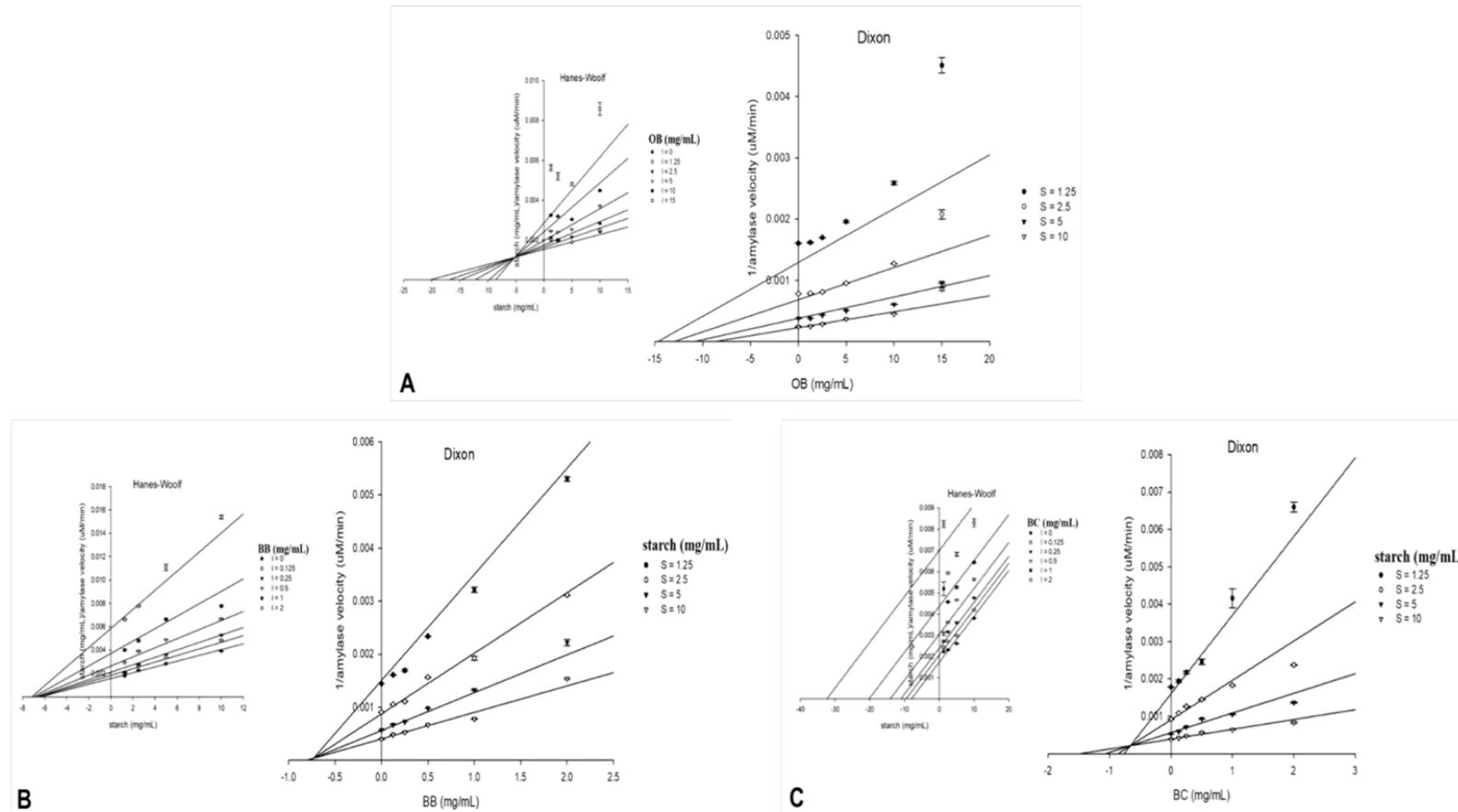


Figure 6.4 Dixon and Hanes-Woolf plot (insets) for α -amylase inhibition by extracts of raw materials

Extracts have illustrated in figures. Various concentrations of extracts are list in the legend entries. (A) OB = oat bran; (B) BB = blueberry powder; (C)

BC = blackcurrant powder.

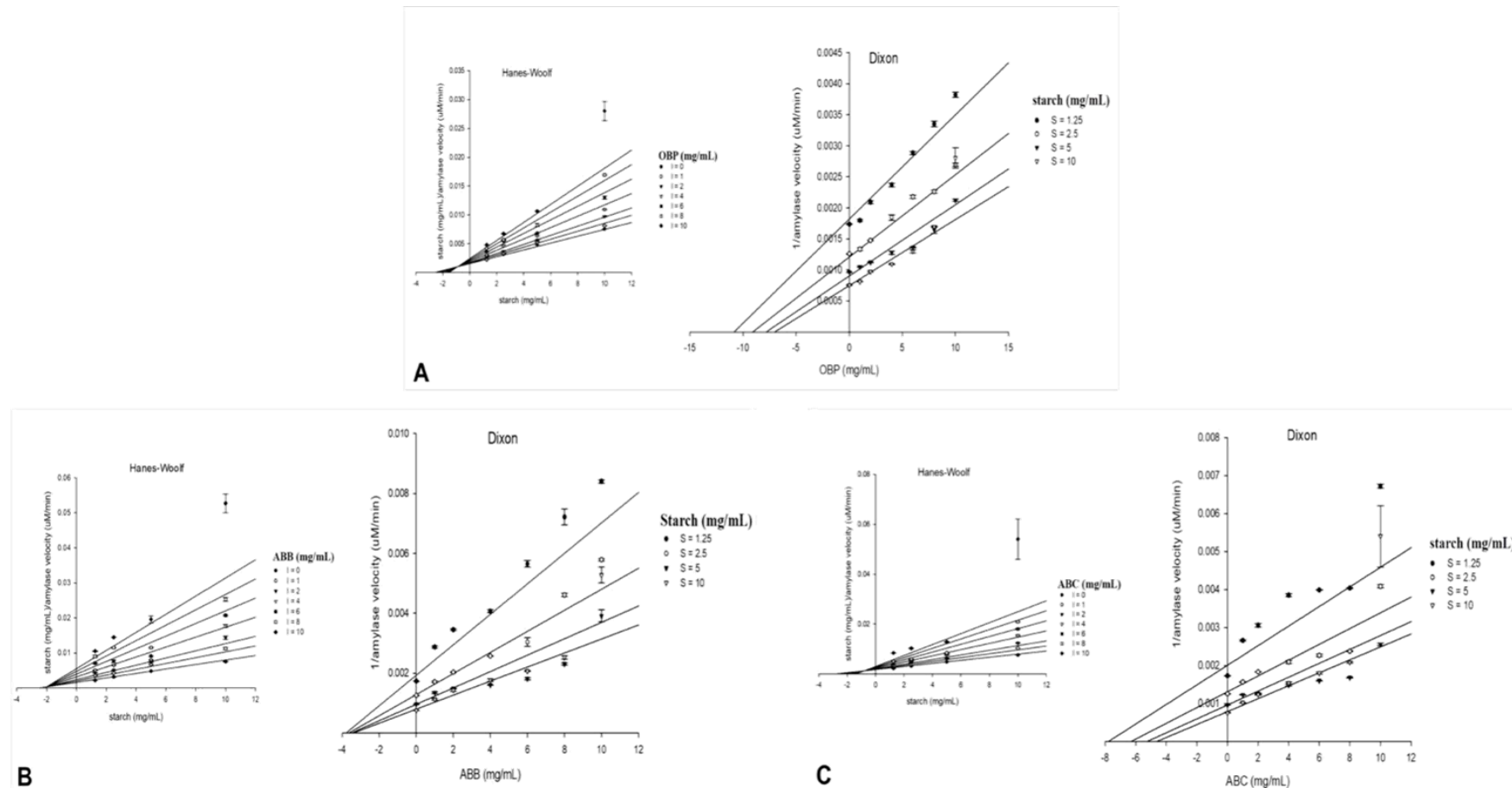


Figure 6.5 Dixon and Hanes-Woolf plot (insets) for α -amylase inhibition by extracts of pastes

Extracts have illustrated in figures. Various concentrations of extracts are list in the legend entries. (A) OBP = oat bran paste; (B) ABB = 25% blueberry enriched oat bran paste; (C) ABC = 25% blackcurrant enriched paste.

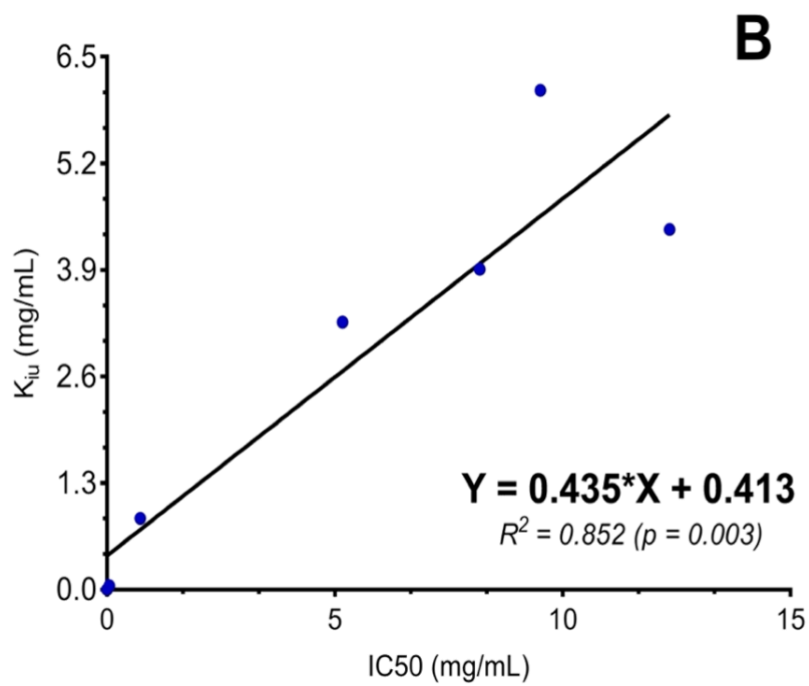
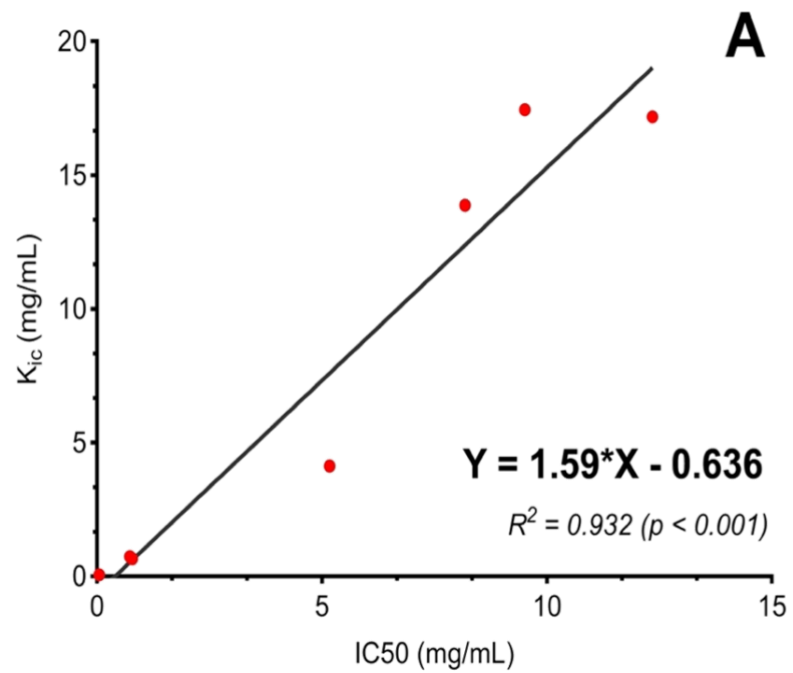


Figure 6.6 Correlations between IC50 and K_{ic} (A), and K_{iu} values (B)

Table 6-3 Alpha-amylase inhibition kinetics constants

Groups	Alpha-amylase inhibition kinetic constants				
	Inhibition type	K _{ic} (mg/mL)	K _{iu} (mg/mL)	1/K _{ic} (mL/mg)	1/K _{iu} (mL/mg)
Acarbose	<i>Mixed</i>	0.06 ± 0.01	0.04 ± 0.00	16.75	22.37
Oat bran	<i>Mixed</i>	17.19 ± 5.22 ^A	4.39 ± 0.76 ^A	0.06	0.23
Blueberry powder	<i>Mixed</i>	0.74 ± 0.09 ^B	0.87 ± 0.03 ^B	1.37	1.15
Blackcurrant powder	<i>Competitive</i>	0.66 ± 0.04 ^B	ND	1.51	ND
Oat bran paste	<i>Mixed</i>	17.45 ± 6.90 ^a	6.09 ± 1.15 ^a	0.06	0.16
25% blueberry enriched paste	<i>Mixed</i>	4.12 ± 1.20 ^b	3.26 ± 0.38 ^b	0.24	0.31
25% blackcurrant enriched paste	<i>Mixed</i>	13.88 ± 8.20 ^a	3.91 ± 1.62 ^b	0.07	0.26

Values are mean ± standard deviation, n = 3. Comparison within the raw materials is expressed by uppercase letters, while comparison within the paste is expressed by small case letters. Values with different letters in the same column are statistically different ($p < 0.05$). ND = no data.

6.3.5 The Lineweaver-Burk plots for the α -amylase inhibitory activity

The Lineweaver-Burk plots of the extract of blackcurrant powder (**Fig. 6-7C**) has an intersection on the y axis, which further demonstrated that the mode of action of the extract from blackcurrant powder on the α -amylase was through competitive inhibition. The intersects of Lineweaver-Burk plots for oat bran extract (**Fig. 6-7B**), blueberry powder extract (**Fig. 6-7D**), and the extracts of pastes (**Fig. 6-8**) were found to have a negative x value, indicating that they might be mixed-type inhibitors of α -amylase. This corresponds with the results of Dixon and Hanes-Woolf plots.

The K_m^{app} and V_{max}^{app} (**Table 6-4**) were obtained from the Michaelis-Menten equation. A K_m^{app} of a typical competitive inhibitor should increase, while its V_{max}^{app} should remain constant (Teixeira *et al.*, 2019). Such observations were shown with the values of the extract from blackcurrant powder. There was some slight decrease in K_m^{app} values of the extract from blueberry powder and the pastes (due to the potential significant errors in plots), however they still showed a general increasing trend. Simultaneously, the decreased V_{max}^{app} were observed of all mixed-type inhibitors since it would take longer to leave the active site for substrates.

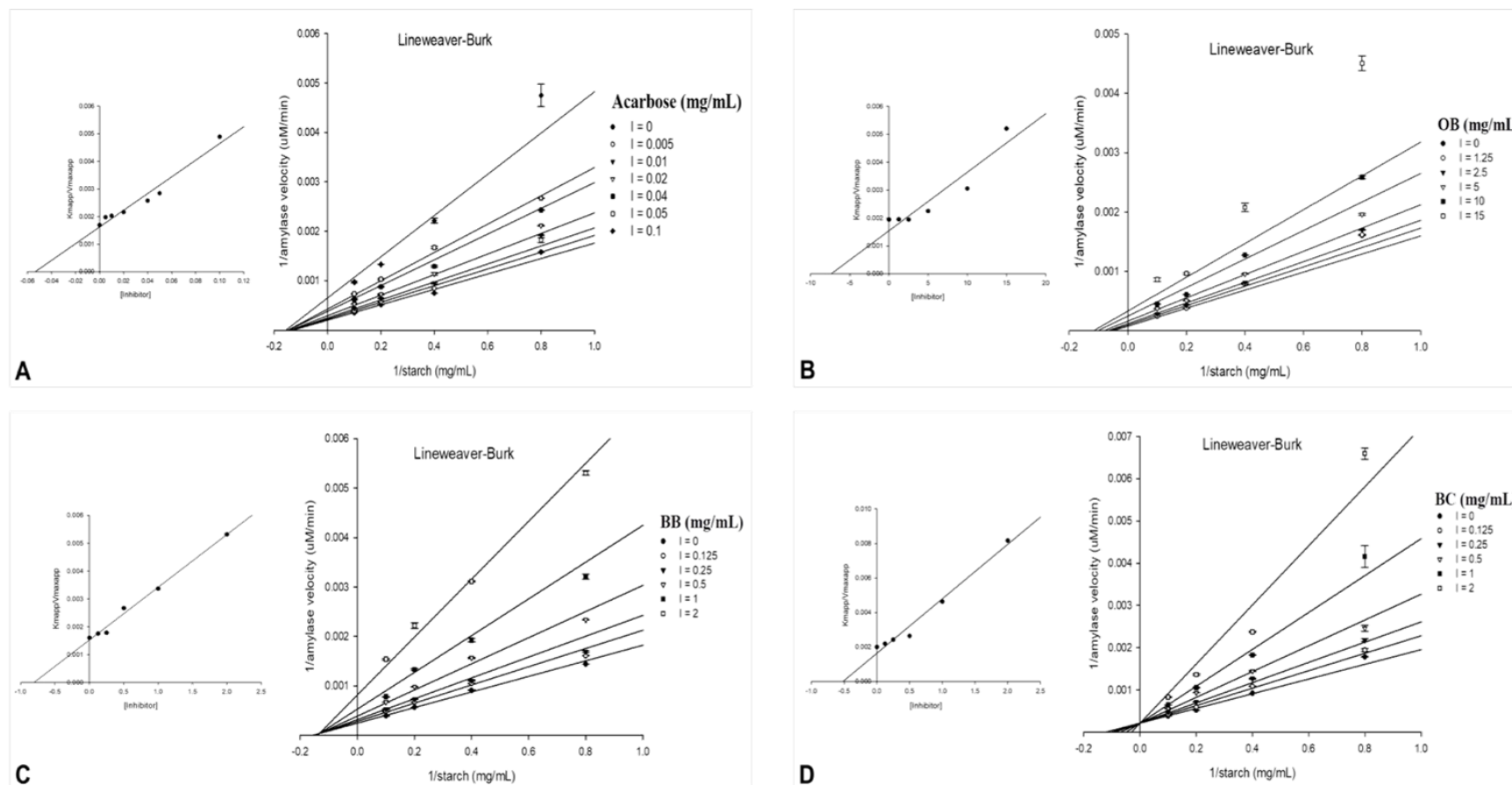


Figure 6.7 Lineweaver-Burk plot and direct linear plot (insets) for α -amylase inhibition by acarbose, and extracts of raw materials

Extracts have illustrated in figures. Various concentrations of extracts are list in the legend entries. (A) Acarbose; (B) OB = oat bran; (C) BB = blueberry powder; (D) BC = blackcurrant powder.

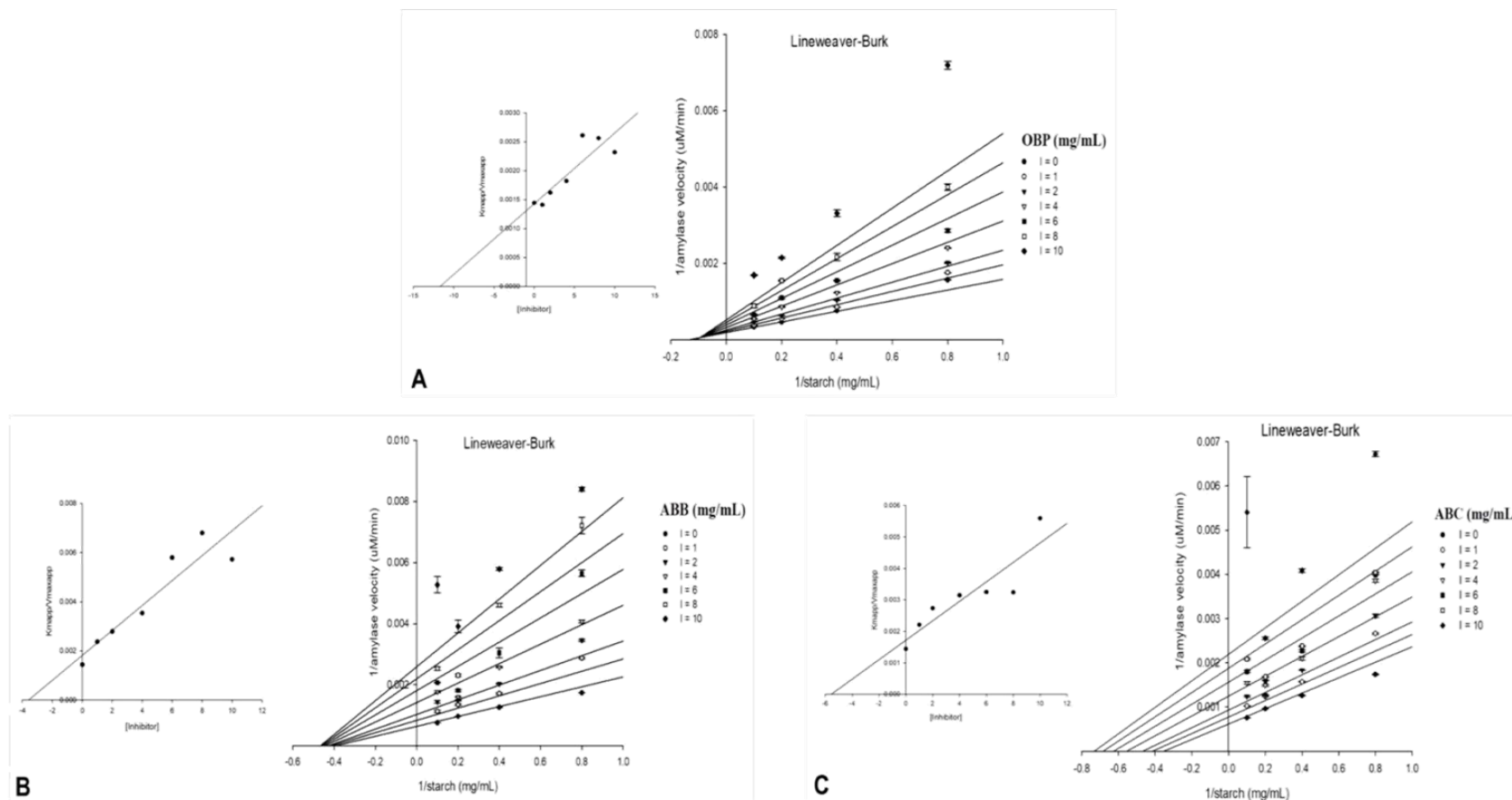


Figure 6.8 Lineweaver-Burk plot and direct linear plot (insets) for α -amylase inhibition by extracts of pastes

Extracts have illustrated in figures. Various concentrations of extracts are list in the legend entries. (A) OBP = oat bran paste; (B) ABB = 25% blueberry powder enriched oat bran paste; (C) ABC = 25% blackcurrant powder enriched oat bran paste.

Table 6-4 Detailed α -amylase inhibition kinetics constants of K_m^{app} and V_{max}^{app}

Groups	K_m^{app} (mg/mL)					V_{max}^{app} (mM/min)						
	A	B	C	D	E	A	B	C	D	E	F	G
Acarbose	9.433	10.360	8.492	7.0730	7.150	5.578	5.245	4.187	3.271	2.774	2.224	
Oat bran	41.290	35.980	31.460	27.940	21.470	21.240	18.440	16.190	12.420	7.026	2.983	
Blueberry powder	6.849	5.693	5.039	6.511	6.575	4.270	3.245	2.822	2.440	1.950	0.9928	
Blackcurrant powder	13.920	10.910	10.410	7.665	25.650	6.948	4.977	4.886	4.897	5.536	4.326	
Oat bran paste	2.266	1.972	2.004	2.002	2.522	1.569	1.399	1.237	1.099	0.9657	0.7698	0.509
25% blueberry enriched paste	2.266	2.682	2.702	2.875	6.705	1.569	1.130	0.967	0.812	1.156	0.542	0.262
25% blackcurrant enriched paste	2.266	2.755	3.359	3.092	2.830	1.569	1.246	1.226	0.982	0.871	0.786	0.446

Data were taken from Lineaweaver-Burk plot. Characters (A to G) represent the concentrations of inhibitors (A = 0; E or G = the highest concentration; Increasing order from A to F or G).

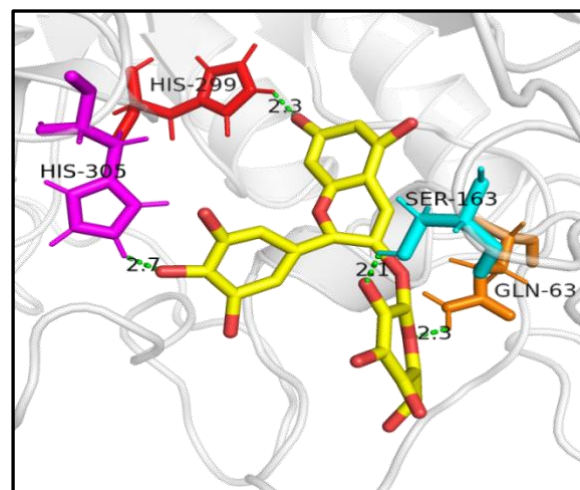
6.3.6 Molecular docking study

To provide a deeper insight into the interaction of α -amylase with anthocyanins, a molecular docking study was employed. Four main anthocyanidins inhibitors were docked into the catalytic site of the α -amylase. **Fig. 6-9** predicts the binding modes of the four main anthocyanins (del-3-glu, cya-3-glu, mal-3-glu, and cya-3-rut) with α -amylase. The model illustrated that the anthocyanins could fit into the binding pocket of the active site (**Table 6-5**). Wu *et al.* (2013) reported that cya-3-glu and del-3-glu effectively aided insulin secretion from rodent pancreatic β -cells *in vitro* compared with the other anthocyanins and anthocyanidins, and their study also demonstrated that the number of hydroxyl groups on the B-ring of anthocyanins played a crucial role in their ability to secrete insulin. The results in this chapter indicate that del-3-glu exhibited the strongest binding affinity of both α -amylase (-10.4 kcal/mol), followed by cya-3-glu. As shown in **Fig. 6-10**, the number of hydroxyl groups in the B-ring of the four anthocyanins are ranked in the following order: del-3-glu > cya-3-glu = cya-3 rut > mal-3-glu, which was consistent with the results of the binding affinities (**Table 6-5**). These results confirmed that anthocyanins had the ability to enter into the active site of the α -amylase, and further inhibited the catalytic action of α -amylase through hydrogen bonding. Thus, the significant α -amylase inhibitory effects of the materials may be due to the number of hydroxyl groups on B-ring. A previous study reported that the addition of disaccharide affects the polarity of anthocyanins and different glycosides alter the chemical structure of anthocyanins, either of which may enhance or decrease the inhibition activity of anthocyanins. Interestingly, although del-3-glu exhibited the weakest binding affinity with α -amylase, the content of mal-3-glu was much lower than other anthocyanins, such as cya-3-rut, which was accounted for the largest group in all extracts of pastes. In terms of the α -amylase inhibitory activity, the extract of 25% blueberry enriched paste showed the strongest inhibitory effect than the other extracts of pastes. Therefore, it can be assumed that a higher

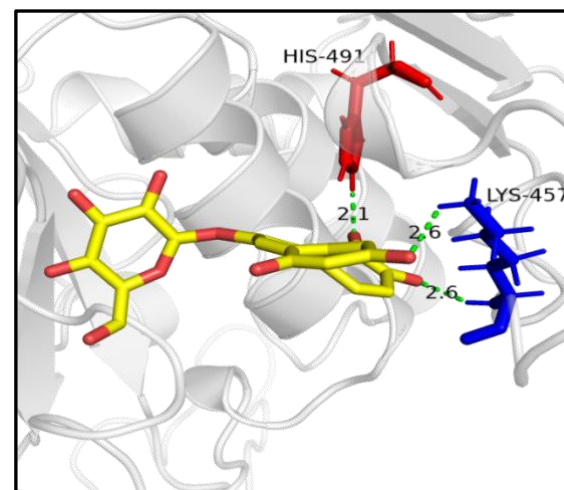
binding affinity does not necessarily result in a greater inhibition activity since the inhibitory activity is influenced by not only binding affinity, but also the chemical structure and the glucoside form of the anthocyanin. Molecular docking studies are typically performed under theoretical vacuum conditions, which varies from actual experimental conditions.

Table 6-5 Predicted binding affinity for anthocyanins present in samples docked with α -amylase

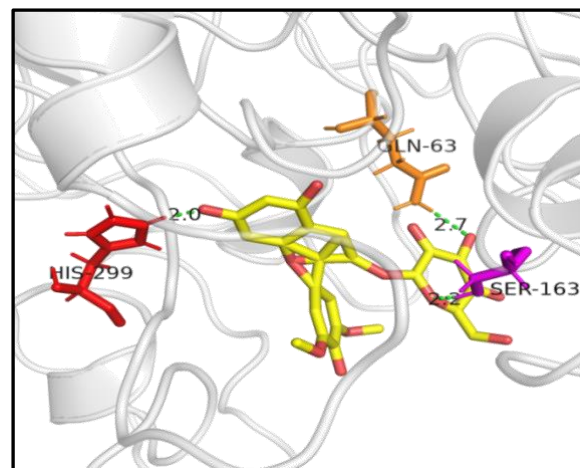
Affinity (kcal/mol)	No. of H-bonds	Amino acid residues
-10.4	4	HIS-305, HIS-299, SER-163, and GLN-63
-9.1	3	HIS-491 and LYS-457
-8.8	2	HIS-299, GLN63, and SER-163
-9.1	5	HIS-299, ARG-195, LYS-200, GLN-63, and ASP-300



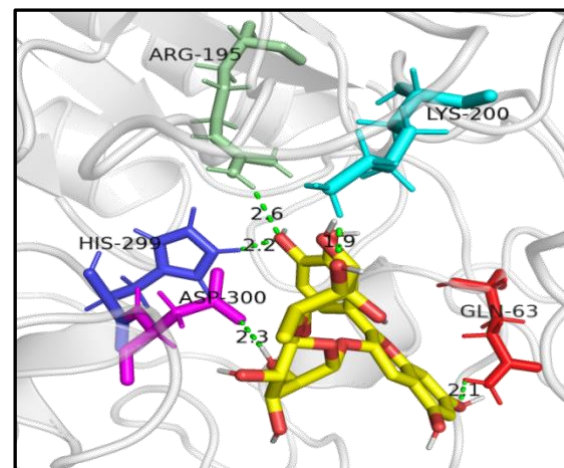
Del-3-glu



Cya-3-glu



Mal-3-glu



Cya-3-

Figure 6.9 Molecular docking studies on the interaction of the anthocyanins with the α -amylase

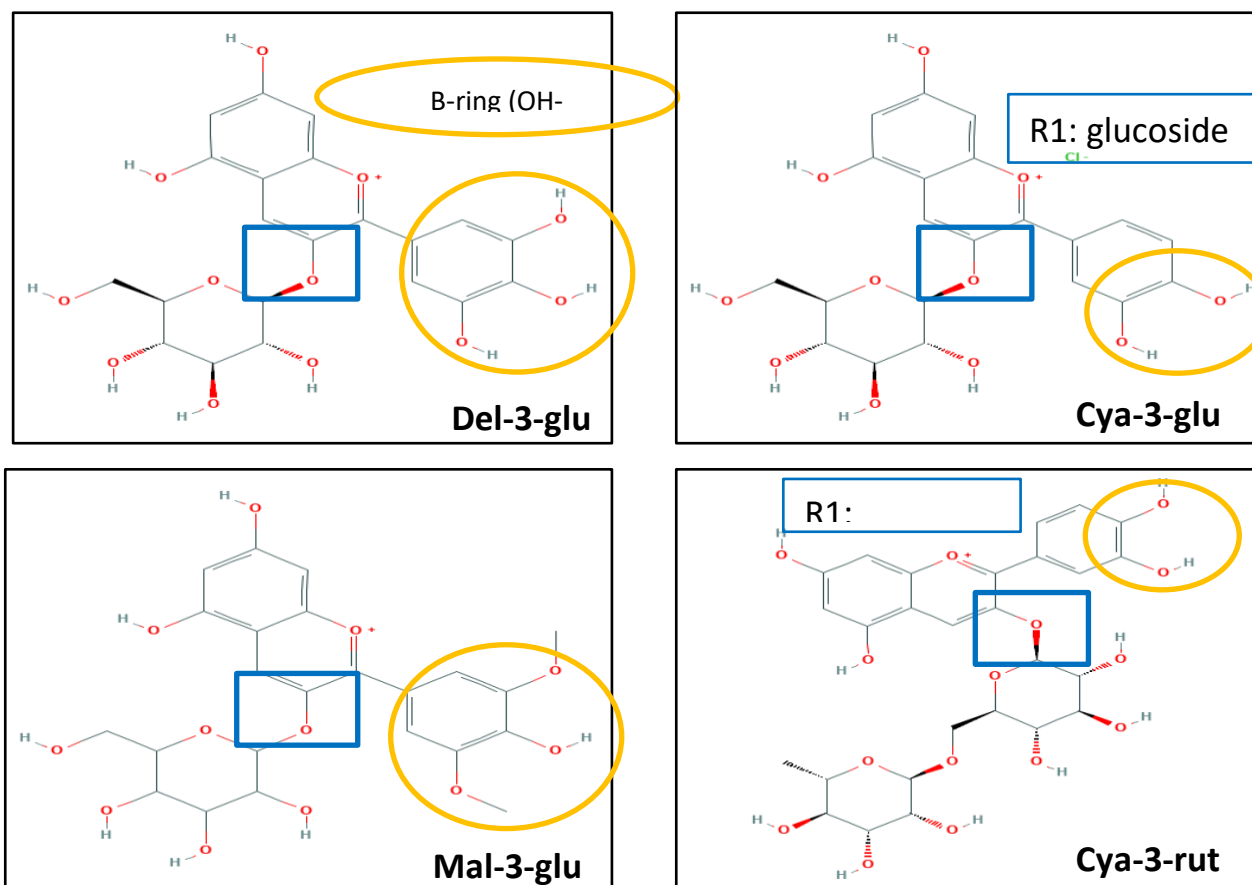


Figure 6.10 Chemical structure of four anthocyanins

Del-3-Glu, Cya-3-Glu, Mal-3-Glu, and Cya-3-Rut (downloaded from PubChem, <https://pubchem.ncbi.nlm.nih.gov>).

6.4 Conclusion

In summary, this chapter identified the major anthocyanin profiles in the extracts of raw materials and pastes, as well as studying their α -amylase inhibitory activities, and the potential mechanisms for the interaction of anthocyanins and α -amylase. The results showed that the main anthocyanins in the extracts of blueberry and blackcurrant enriched pastes were cyanidin and delphinidin. The extract of blueberry enriched paste exhibited a higher α -amylase inhibitory effect than the extract from blackcurrant enriched paste and pure oat bran paste. According to the α -amylase kinetic analysis, it was found that the extracts from blueberry and blackcurrant enriched pastes acted as mixed inhibitors against α -amylase. Molecular docking studies revealed that the inhibition of α -amylase by anthocyanins occurred due to the attachment of anthocyanins to the active site of α -amylase, which has the catalytic centre of the enzymes in order to restrict the entry of substrates. Due to the advantages of more hydroxyl groups in the B-ring, anthocyanins obtained from the extracts of blueberry and blackcurrant enriched pastes could be developed as potential α -amylase inhibitors. Hence, foods rich in cyanidin and/or delphinidin might be useful in the prevention and management of T2DM.

Chapter 7

Evaluation of the *in vitro* inhibitory activity towards α -glucosidase of the extracts from blueberry and blackcurrant powders enriched oat bran paste

Abstract

The α -glucosidase inhibitory activities of extracts from oat bran, blueberry and blackcurrant powder, and oat bran paste enriched with 25% blueberry or blackcurrant powder, were studied in this chapter by measuring their half inhibitory (IC₅₀) concentrations. The potential inhibitory mechanisms were also investigated by inhibition kinetics and molecular docking studies. The results showed that the extract of blackcurrant powder exhibited the strongest α -glucosidase inhibitory activity with an IC₅₀ of 8.99 mg/mL. The extract of blackcurrant enriched oat bran paste showed stronger α -glucosidase inhibitory activity compared to the extract of blueberry enriched paste, and both were competitive inhibitors, while the other extracts were mixed-type inhibitors. Alpha-glucosidase inhibitory activities were potentially driven by hydrogen bonding. Cyanidin-3-glucoside and delphinidin-3-glucoside had a stronger binding affinity compared to malvidin-3-glucoside and cyanidin-3-rutinside. These results showed that the efficiency of α -glucosidase inhibitory activity of the different food matrices of blueberry and blackcurrant enriched oat bran paste depends on the site of action, the mechanisms, and binding affinities. Synergies occurring in the food matrix enhanced their inhibitory activity. The inhibitory activity of the berry-enriched cereal food products towards α -glucosidase gives a potential for T2DM.

Keywords: anthocyanins, α -glucosidase, inhibition kinetics, molecular docking

7.1 Introduction

T2DM is a metabolic disease, which can result from factors involved in poor lifestyles, such as smoking, a high-carbohydrate diet, and reduced physical inactivity (Magkos, Yannakoulia, Chan, & Mantzoros, 2009). A possible intervention for T2DM is by inhibiting the carbohydrate hydrolysing enzymes that metabolise starch molecules to glucose molecules. Diets rich in fruit and whole grain cereal have been demonstrated to reduce the risk of T2DM (Khazrai, Defeudis, & Pozzilli, 2014).

Salivary and pancreatic α -amylase digest dietary starch into reducing sugars. Subsequently, α -glucosidases break the oligo- and disaccharides down into the single glucose molecules in the upper intestine (Navarro, Abelilla, & Stein, 2019; Peyrot des Gachons & Breslin, 2016). The inhibition of α -glucosidase prevents the degradation of oligosaccharides into the glucose, and decreases the glucose which is available to be absorbed into the blood from the small intestine (Gangoiti *et al.*, 2020). Alpha-glucosidase inhibitors have been shown to reduce the postprandial blood glucose spike mainly through binding to the active sites of the substrate and hydrolysing the α (1 \rightarrow 4) bonds in carbohydrate compounds (Lattimer & Haub, 2010). Acarbose is a well-known strong α -glucosidase inhibitor and is considered as a last-line of intervention treatment for T2DM. However, it has some severe side effects, such as hepatotoxicity (Hanefeld & Schaper, 2007; Yilmazer-Musa, Griffith, Michels, Schneider, & Frei, 2012).

Several studies have reported the inhibitory effect of phytochemicals, particularly phenolic compounds in berry fruits, on the carbohydrate hydrolysing enzymes, namely α -glucosidases, but without the side effects observed in acarbose (Brown, Anderson, Racicot, Pilkenton, & Apostolidis, 2017; Caruso *et al.*, 2019; Khan *et al.*, 2016). Polyphenols are one of the main phytochemicals in fruits and exhibit antioxidants in the diet. Polyphenols contain at least one

aromatic ring with hydroxyl groups and another substituent. Polyphenols are further classified into flavonoids and non-flavonoids. Anthocyanidins are the main sub-groups found within the flavonoid category (Tsao, 2010). Flavonoids are well documented inhibitors towards α -glucosidase and can reduce or retard glucose uptake in the small intestine, via prevention of digestion of the disaccharide. This property could be used to potentially prevent and treat T2DM (Alkhalidy, Wang, & Liu, 2018; Shi *et al.*, 2019).

Importantly, many studies have already shown that the intake of plant material rich in polyphenols can cause anti-hyperglycaemic effects in animals, possibly via α -glucosidase and/or α -amylase inhibition (Li *et al.*, 2018; Oboh *et al.*, 2014; Yang *et al.*, 2019). However, phenolic compounds can also interfere with glucose uptake at the interface of the brush border. Some studies have also reported a reduced inhibitory activity when eating berry fruits, which was possibly due to the differences in the food matrix or the dose. Therefore, further human intervention studies are still needed (Boath, Grussu, Stewart, & McDougall, 2012).

The phenolic compounds in food products are believed to behave synergistically with one another (Das, Bhaumik, Raychaudhuri, & Chakraborty, 2012; Quero, Mármol, Cerrada, & Rodríguez-Yoldi, 2020). As the beneficial effects are usually synergistic, it is not uncommon to observe reduction of bioactivity when performing fractionation-guided bioactivity assays. Oat bran has also been shown to reduce postprandial glucose levels and body mass index (Ulmius *et al.*, 2011). It is conceivable that the combination of berry fruits with oat bran may have the potential to show a higher the α -glucosidase inhibitory activity, when compared with the consumption of berry fruits and cereals individually. In Chapter 6, the main anthocyanins in the extracts of blueberry and blackcurrant enriched oat bran paste were identified, and the α -amylase inhibitory activities were studied. The results showed that the bioactive compounds, especially the anthocyanins, from the extracts of blueberry powder and blackcurrant powder increased the α -amylase inhibitory activity of oat bran paste. The results suggested that the

extract from blueberry powder exhibited higher α -amylase inhibitory than the extract from blackcurrant powder.

The focus of this chapter is on the effect of blueberry powder and blackcurrant powder on the α -glucosidase inhibitory activity of the oat bran paste. Based on the results of chapter 6, a 25% enrichment with blueberry and blackcurrant powder was chosen as the additional level for oat bran, to research the synergistic effect on the inhibition of α -glucosidase activity. The Dixon, Hanes-Woolf, and Lineweaver-Burk plots were used to determine the types of inhibition. In addition, a molecular docking study, on the interaction of α -glucosidase and the four major anthocyanins, was simulated to show the potential mechanisms of the inhibitory activity against α -glucosidase.

7.2 Methods

7.2.1 Preparation of the oat bran paste

Pastes were prepared as described in 3.2.

7.2.2 Extraction of the raw materials and pastes

The extraction of samples was performed as outlined in 3.5.

7.2.3 Alpha-glucosidase inhibitory activity

The α -glucosidase inhibitory activity was performed followed by the method in 3.14.3.

7.2.4 Alpha-glucosidase inhibitory kinetics of extracts

The kinetics of α -glucosidase inhibition by extracts were determined as described in 3.14.4.

7.2.5 Molecular docking study on α -glucosidase

The molecular docking study on α -glucosidase was employed as outlined in 3.15.

7.2.6 Statistical analysis

Statistical analysis was carried out as outlined in 3.17.

7.3 Results and discussion

7.3.1 Alpha-glucosidase inhibitory activity

Previous studies have demonstrated a correlation between the inhibitors of α -glucosidase and blood glucose regulation in T2DM (Pałasz *et al.*, 2019). Inhibitors of α -glucosidase slow the degradation of carbohydrates in the small intestine and limit postprandial blood glucose spike. Thus, inhibition of glycosidases has a significant effect on the metabolism of polysaccharides, raising the scope for discovery and the development of new therapeutic agents against T2DM and obesity (Proença *et al.*, 2017). Owing to their similarity with disaccharides or oligosaccharides in molecular structure, most α -glucosidase inhibitors can attach to the carbohydrate binding site of α -glucosidase. The inhibitor-glucosidase complexes have a stronger affinity than the carbohydrate-glucosidase complexes (Assefa *et al.*, 2019). Non-competitive, uncompetitive, and mixed inhibition actions of α -glucosidase inhibitors from flavonoid-based compounds have been reported (Abuelizz *et al.*, 2019; Brás, Cerqueira, Ramos, & Fernandes, 2014). The inhibition of α -glucosidase involved in the digestion of carbohydrates can significantly decrease the postprandial blood glucose peak after consumption of a mixed carbohydrate diet, and this has been shown to be important in preventing the progression of impaired glucose tolerance towards T2DM (Blaak *et al.*, 2012). **Fig. 7-1** illustrates the inhibitory effects, including the positive control, acarbose, on α -glucosidase. The results showed that as the concentration of inhibitor increased, the activity of α -glucosidase decreased in a concentration-dependent manner. Contrary to the α -amylase inhibitory results, blackcurrant powder extract showed a stronger inhibitory effect ($IC_{50} = 8.99$ mg/mL) on α -glucosidase when compared to blueberry powder extract ($IC_{50} = 10.90$ mg/mL)

($p < 0.01$) (**Fig. 7-1D**). Oat bran extract showed the weakest effect of all the raw materials extracts ($IC_{50} = 46 \text{ mg/mL}$) ($p < 0.01$). A similar pattern was found among the extracts of pastes, the extract of 25% blackcurrant enriched paste ($IC_{50} = 35.80 \text{ mg/mL}$) presented the highest α -glucosidase inhibitory effect, followed by the extract of 25% blueberry enriched paste ($IC_{50} = 68.30 \text{ mg/mL}$). The extract of pure oat bran paste exerted the lowest inhibitory effect, with its IC_{50} value being nearly 5- fold higher than the value of the extract from 25% blackcurrant enriched paste ($p < 0.01$). The inhibitory effects of all inhibitors on α -glucosidase were in the following order: Acarbose > blackcurrant powder extract > blueberry powder extract > 25% blackcurrant enriched paste extract > oat bran extract > 25% blueberry enriched paste extract > oat bran paste extract.

7.3.2 The Michaelis-Menten curves for the α -glucosidase inhibitory activity

The Michaelis-Menten equation was employed to study the detailed kinetic characterisation of the α -glucosidase inhibitory activity, V_{\max} and K_m . The enzyme velocity (v), in the absence and presence of inhibitor, as presented by the Michaelis-Menten curve are shown in **Fig. 7-2**. The Michaelis-Menten curve showed that under the same p-NPG concentration, v decreased with increasing concentration of inhibitor, illustrating that the α -glucosidase inhibitory effect of the inhibitors was in an inhibitor concentration dependent manner. The extract of blueberry and blackcurrant powder showed higher enzymes inhibitory activity compared to oat bran extract ($p < 0.01$), while the extract of 25% blueberry and 25% blackcurrant enriched oat bran paste exhibited stronger enzyme inhibitory effects compared to the extract of oat bran paste ($p < 0.01$).

Higher levels of binding between the inhibitors with α -glucosidase also triggered an increase in the α -glucosidase binding with p-NPG. Thus, the inhibitory activity followed a dose-dependent manner since these inhibitors might be adsorbed by the p-NPG. The increased enzyme binding in the presence of the inhibitors may result from parts of the adsorbed

inhibitors carrying the enzyme. Although these inhibitors could increase the binding of α -glucosidase with the substrates, the initial digestion velocity of p-NPG in the presence of inhibitors was still lower than that without inhibitors. This shows that the inhibition effect of these extracts on α -glucosidase was more obvious than the promoting effect on the enzyme binding with the substrates. At the same concentration of the substrate, the velocity of the α -glucosidase decreased with the increasing level of inhibitor.

The inhibitory activities of these extracts were dose-dependent manners. More enzyme binding in the presence of anthocyanins may result from parts of the adsorbed polyphenols carrying the enzyme. However, although these extracts increased the binding of α -glucosidase with substrates, the initial digestion velocity of substrates in the presence of inhibitors was still lower than that without inhibitor. This revealed that the inhibition effects of phenolic compounds on α -glucosidase were more obvious than the promoting effects on the enzyme binding with substrate.

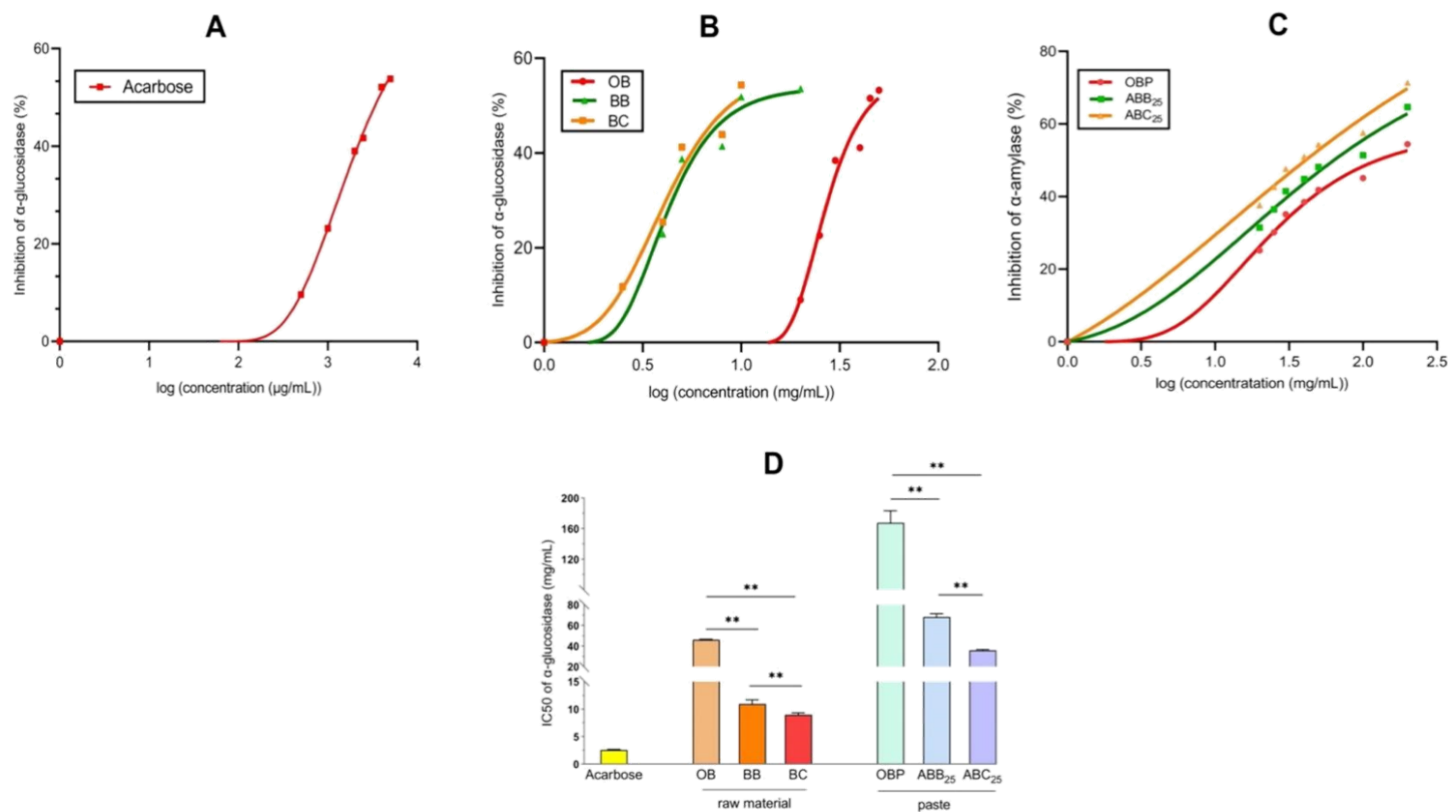


Figure 7.1 Inhibition curves and IC₅₀ of inhibitors against α -glucosidase

The inhibition α -amylase curves by acarbose (1A), extracts of raw materials (1B) and pastes (1C) with different concentrations; IC₅₀ of all inhibitors against α -glucosidase (1D). OB = oat bran; BB = blueberry powder; BC = blackcurrant powder; OBP = oat bran paste; ABB₂₅ = 25% blueberry enriched oat bran paste; ABC₂₅ = 25% blackcurrant enriched oat bran paste.

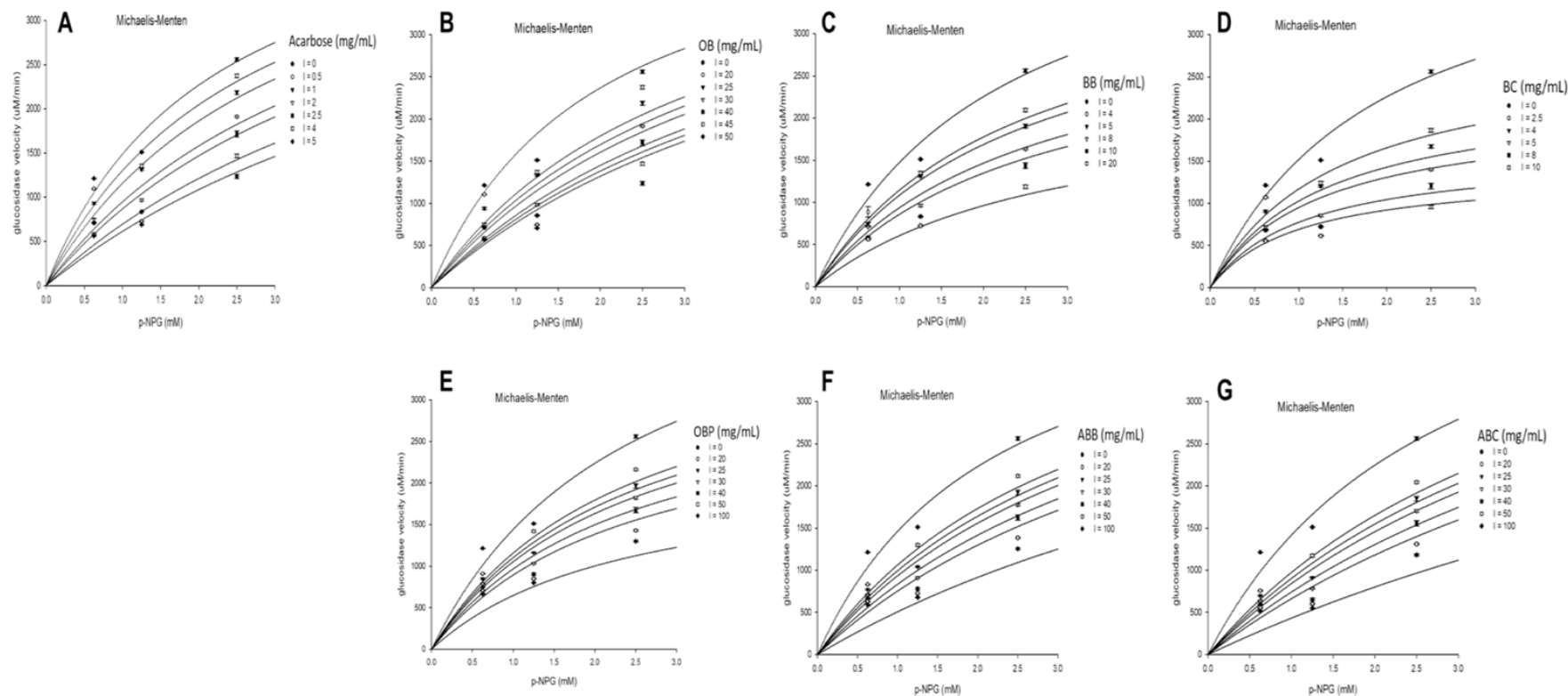


Figure 7.2 Michaelis-Menten for α -glucosidase inhibition by inhibitors

Inhibitors have illustrated in figures. Various concentrations of inhibitors are list in the legend entries. OB = oat bran; BB = blueberry powder; BC = blackcurrant powder; OBP = oat bran paste; ABB = 25% blueberry enriched oat bran paste; ABC = 25% blackcurrant enriched oat bran paste.

7.3.3 The Dixon & Hanes-Woolf plots for the α -glucosidase inhibitory activity

The Dixon plot for acarbose (**Fig. 7-3A**), the extract of 25% blueberry enriched paste (**Fig. 7-4B**), and 25% blackcurrant enriched paste (**Fig. 7-4C**) show clear intersections. However, the Hanes-Woolf plots show parallel lines. This suggests that the mode of action of acarbose and the extract of 25% blueberry and 25% blackcurrant enriched paste is through the competitive inhibition via binding to the active sites of α -glucosidase. **Table 7-1** shows the detailed kinetics parameters of the enzyme. **Table 7-1** illustrates that the $1/K_{ic}$ values of 25% blueberry enriched paste extract (0.0273 mL/mg) was lower than 25% blackcurrant enriched paste extract (0.0308 mL/mg) ($p < 0.05$). The extract of oat bran paste showed the lowest $1/K_{ic}$ value ($p < 0.01$), which was in agreement with the corresponding IC_{50} values presented in **Fig. 7-1D**. The Dixon and Hanes-Woolf plots of oat bran extract (**Fig. 7-3B**), blueberry powder extract (**Fig. 7-3C**), blackcurrant powder extract (**Fig. 7-3D**), and oat bran paste extract (**Fig. 7-4A**) intersected at one point, revealing that their modes of action were all mixed-type. Therefore, they were able to compete with the p-NPG not only in binding with the α -glucosidase, but also in binding with the α -glucosidase-p-NPG complex, forming an 'inhibitor- α -glucosidase-p-NPG' complex. The $1/K_{ic}$ values of the extract of oat bran, blueberry and blackcurrant powder were higher than the corresponding $1/K_{iu}$ values ($p < 0.01$), suggesting that they more likely attached to the active sites of α -glucosidase than non-active sites. The order of K_{ic} values was consistent with IC_{50} values. Strong correlations between IC_{50} and both K_{ic} ($R^2 = 0.973$, $p = 0.001$) and K_{iu} ($R^2 = 0.969$, $p = 0.01$) were also observed in **Fig. 7-5**. These results indicated that the extract of 25% blackcurrant enriched paste was the strongest α -glucosidase inhibitor among all the extracts of pastes ($p < 0.01$). Moreover, according to the previous identification of the major anthocyanins present in extracts of raw materials (**Table 6-1**), and pastes (**Table 6-2**), cyanidin and delphinidin could be the main contribution to its inhibitory effect.

7.3.4 The Lineweaver-Burk plots for the α -glucosidase inhibitory activity

The Lineweaver-Burk plots of acarbose (**Fig. 7-6A**), the extracts of 25% blueberry enriched paste (**Fig. 7-7B**), and the extracts from 25% blackcurrant enriched paste (**Fig. 7-7C**) have an intersection on the y axis, which further illustrates that the mode of action on the α -glucosidase was through competitive inhibition. The intersects of Lineweaver-Burk plots for oat bran extract (**Fig. 7-6B**), blueberry powder extract (**Fig. 7-6C**), blackcurrant powder extract (**Fig. 7-6D**), and oat bran paste extract (**Fig. 7-7A**) were found to have a negative x value, indicating that they might be mixed-type inhibitors of α -glucosidase. This corresponds with the results of Dixon and Hanes-Woolf plots. **Table 7-2** shows the K_m^{app} and V_{max}^{app} values of inhibitors for α -glucosidase inhibition. Generally, K_m^{app} values increased with increasing concentration, whereas the V_{max}^{app} values decreased with increasing concentration.

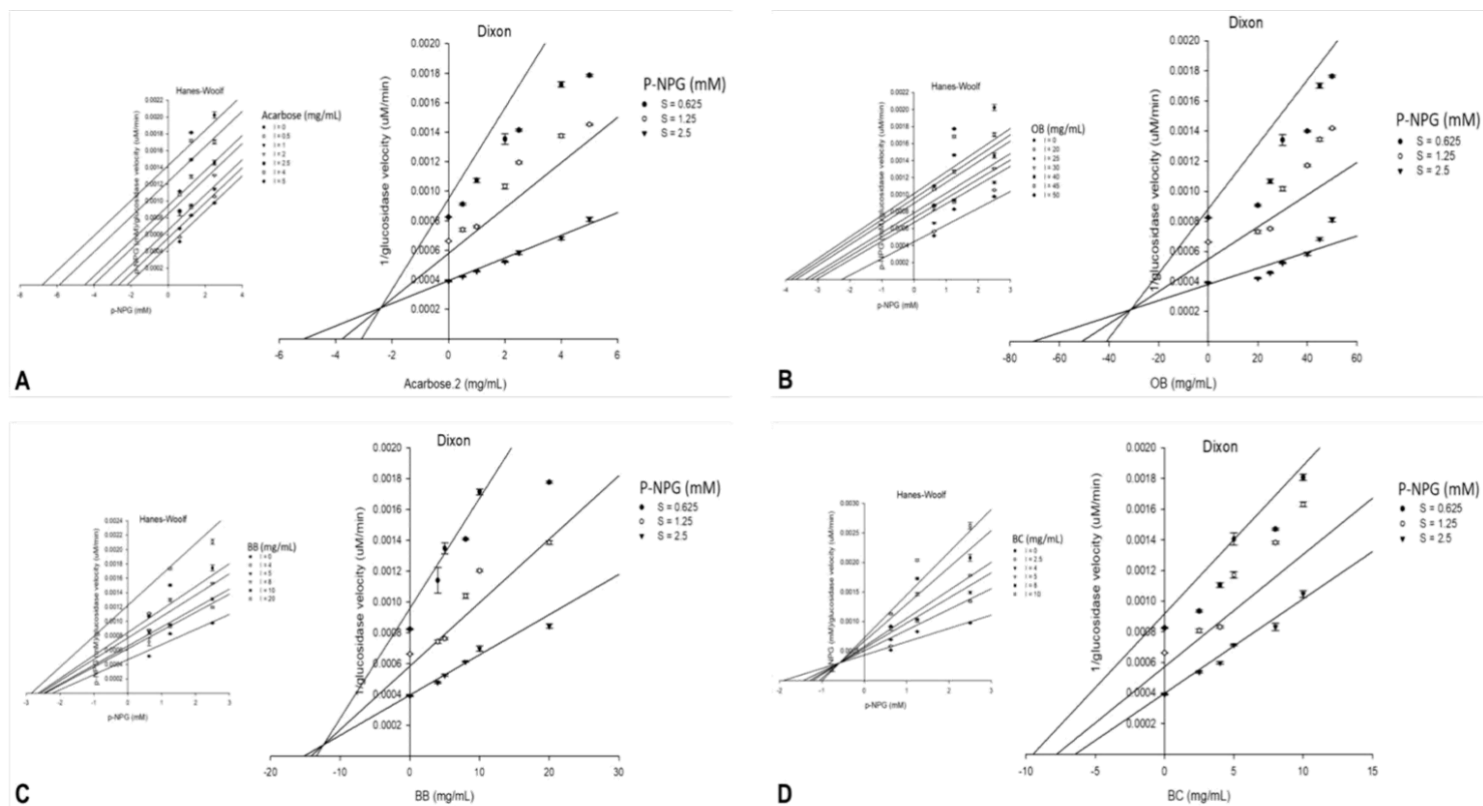


Figure 7.3 Dixon and Hanes-Woolf plot (insets) for α -glucosidase inhibition by acarbose and extracts of raw materials

Inhibitors have illustrated in figures. Various concentrations of inhibitors are list in the legend entries. (A) Acarbose; (B) OB = oat bran; (C) BB = blueberry powder; (D) BC = blackcurrant powder.

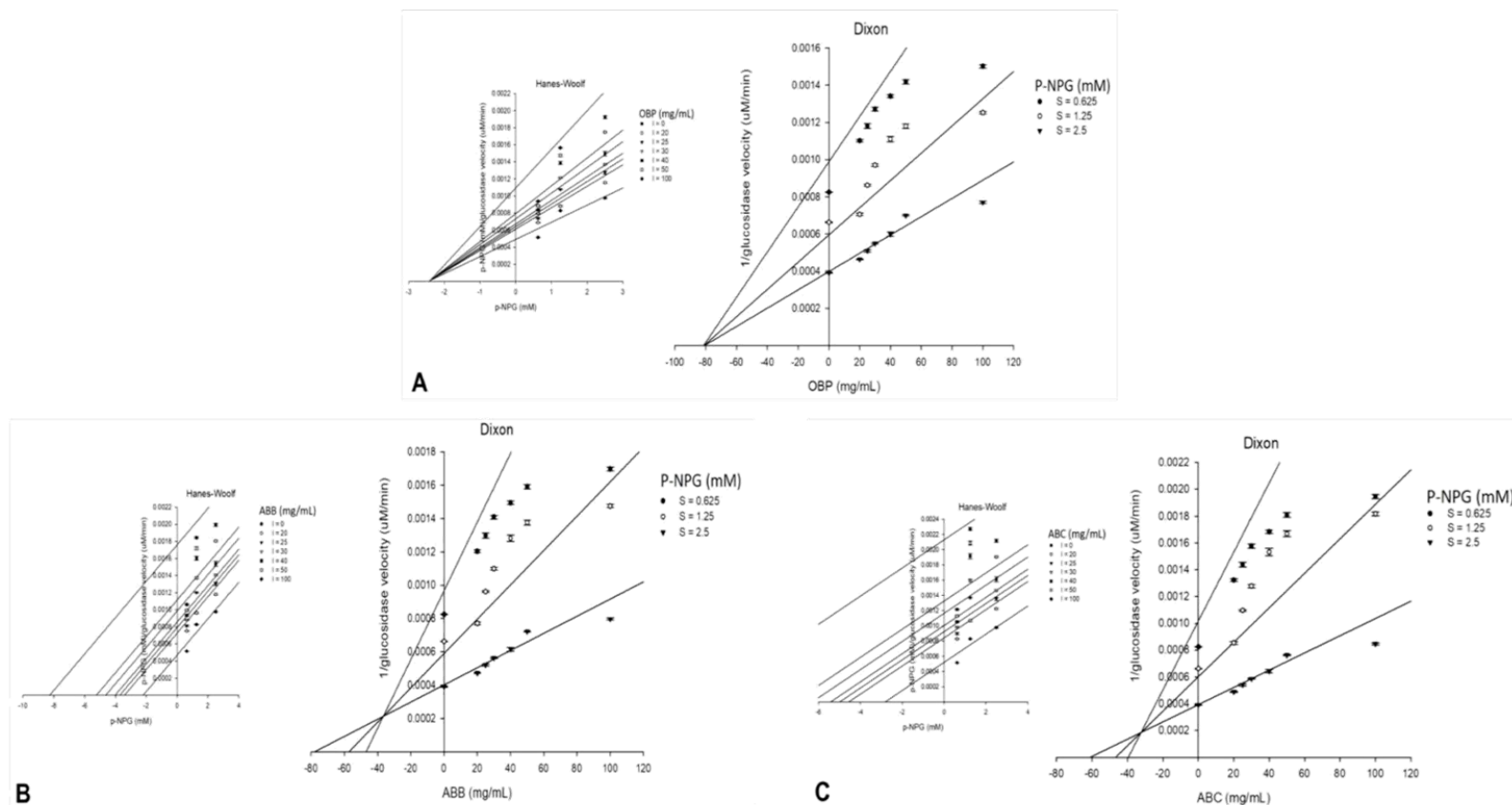


Figure 7.4 Dixon and Hanes-Woolf plot (insets) for α -glucosidase inhibition by extracts of pastes

Inhibitors have illustrated in figures. Various concentrations of inhibitors are list in the legend entries. (A) OBP = oat bran paste; (B) ABB = 25% blueberry enriched oat bran paste; (C) ABC = 25% blackcurrant enriched oat bran paste.

Table 7-1 Alpha-glucosidase inhibition kinetics constants

Groups	α -glucosidase inhibition kinetic constants				
	Inhibition type	K _{ic} (mg/mL)	K _{iu} (mg/mL)	1/K _{ic} (mL/mg)	1/K _{iu} (mL/mg)
Acarbose	<i>Competitive</i>	2.41 ± 0.23	ND	0.42	ND
Raw material					
Oat bran	<i>Mixed</i>	39.33 ± 3.94 ^A	169.86 ± 33.33 ^A	0.03	0.00
Blueberry powder	<i>Mixed</i>	12.43 ± 3.05 ^B	19.06 ± 3.04 ^B	0.08	0.05
Blackcurrant powder	<i>Mixed</i>	14.83 ± 7.36 ^B	4.51 ± 1.58 ^C	0.07	0.22
Paste					
Oat bran paste	<i>Mixed</i>	81.82 ± 28.95 ^a	79.83 ± 22.47	0.01	0.01
25% blueberry enriched paste	<i>Competitive</i>	36.57 ± 1.20 ^b	ND	0.03	ND
25% blackcurrant enriched paste	<i>Competitive</i>	32.39 ± 3.78 ^b	ND	0.03	ND

Values are mean ± standard deviation, n = 3. Values with different letters are statistically different ($p < 0.05$). ND = no data.

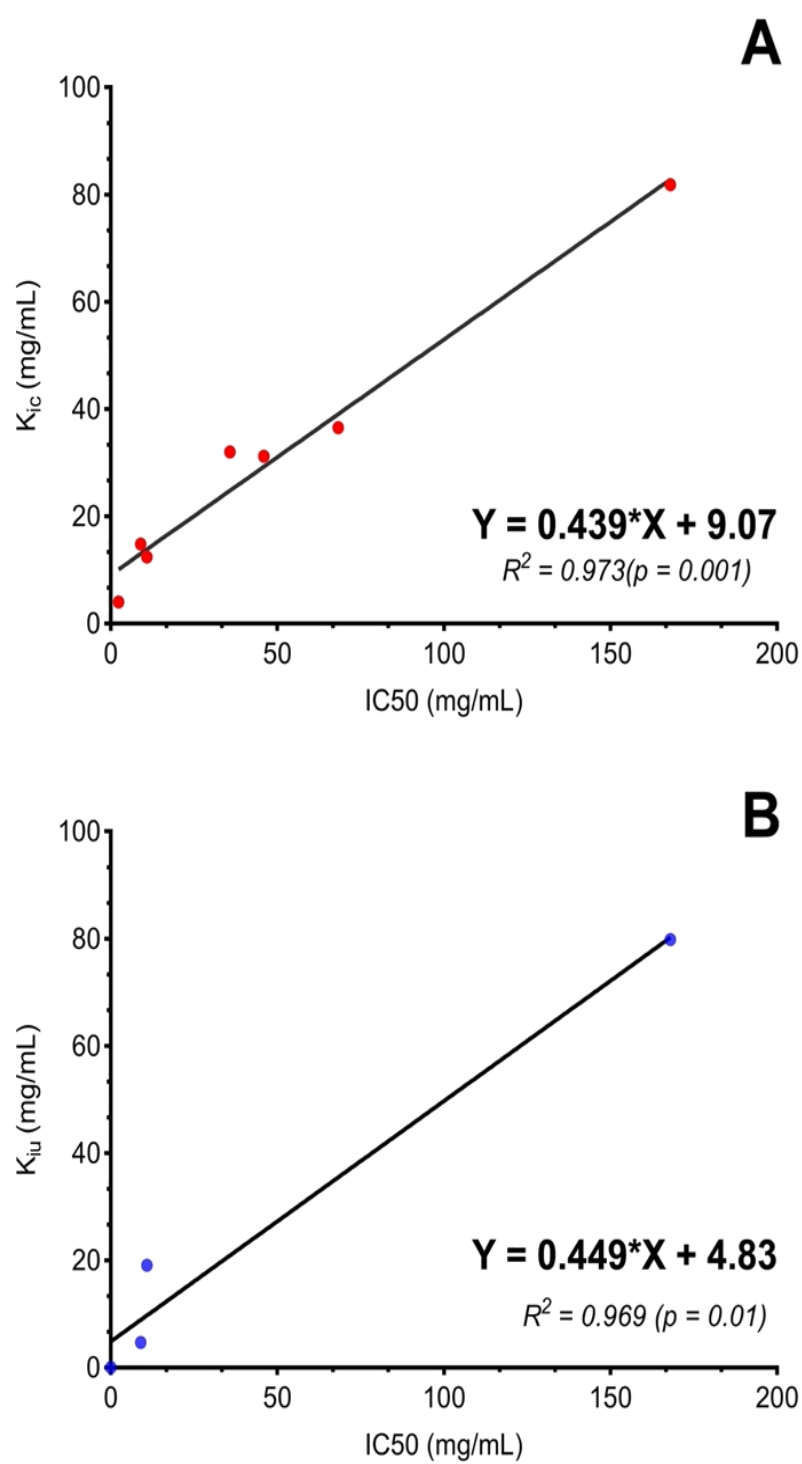


Figure 7.5 Correlations between IC_{50} and K_{ic} (A), and K_{iu} values (B)

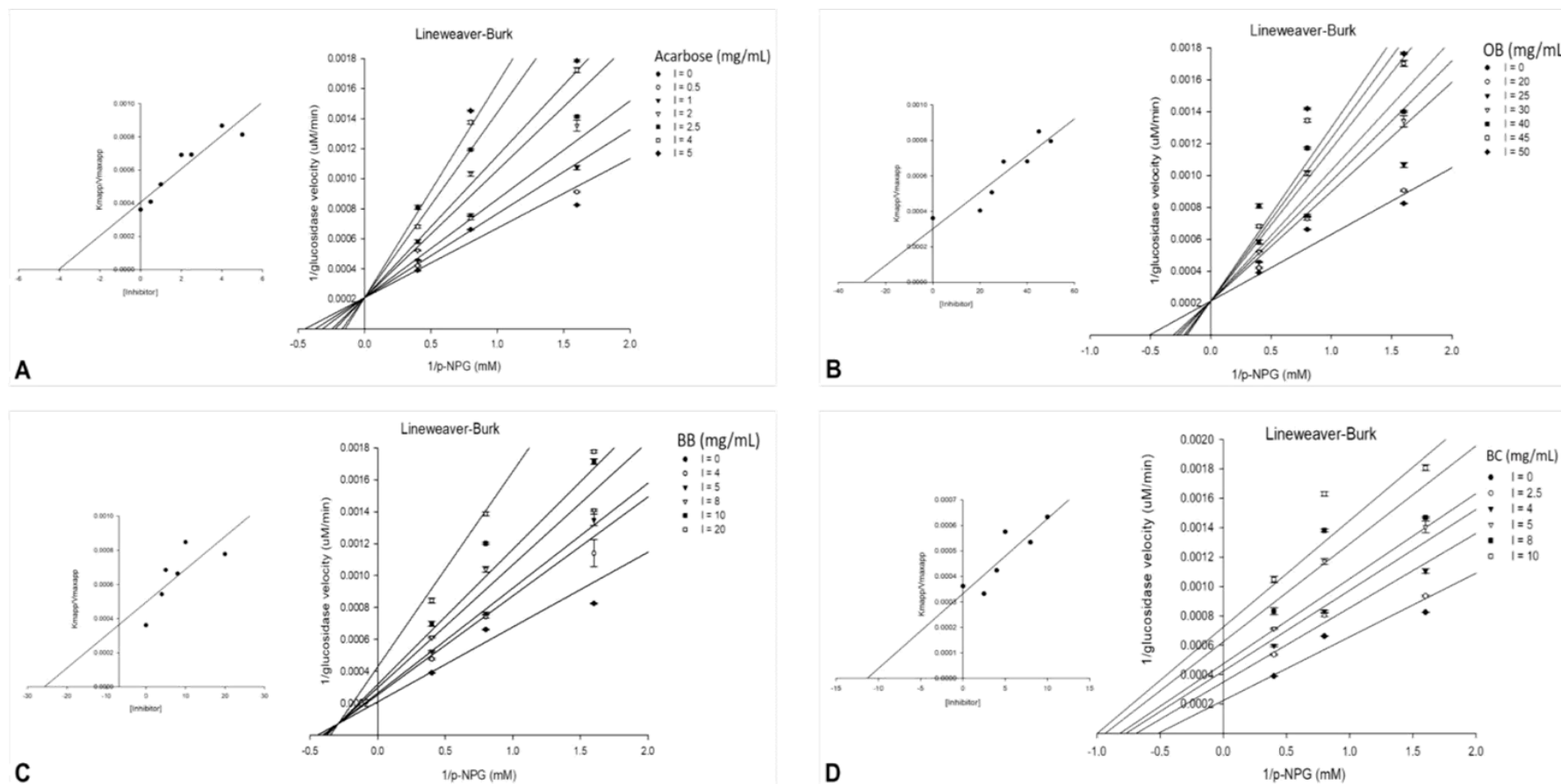


Figure 7.6 Lineweaver-Burk plot and direct linear plot (insets) for α -glucosidase inhibition by acarbose and extracts of raw materials

Inhibitors have illustrated in figures. Various concentrations of inhibitors are list in the legend entries. (A) Acarbose; (B) OB = oat bran; (C) BB = blueberry powder; (D) BC = blackcurrant powder.

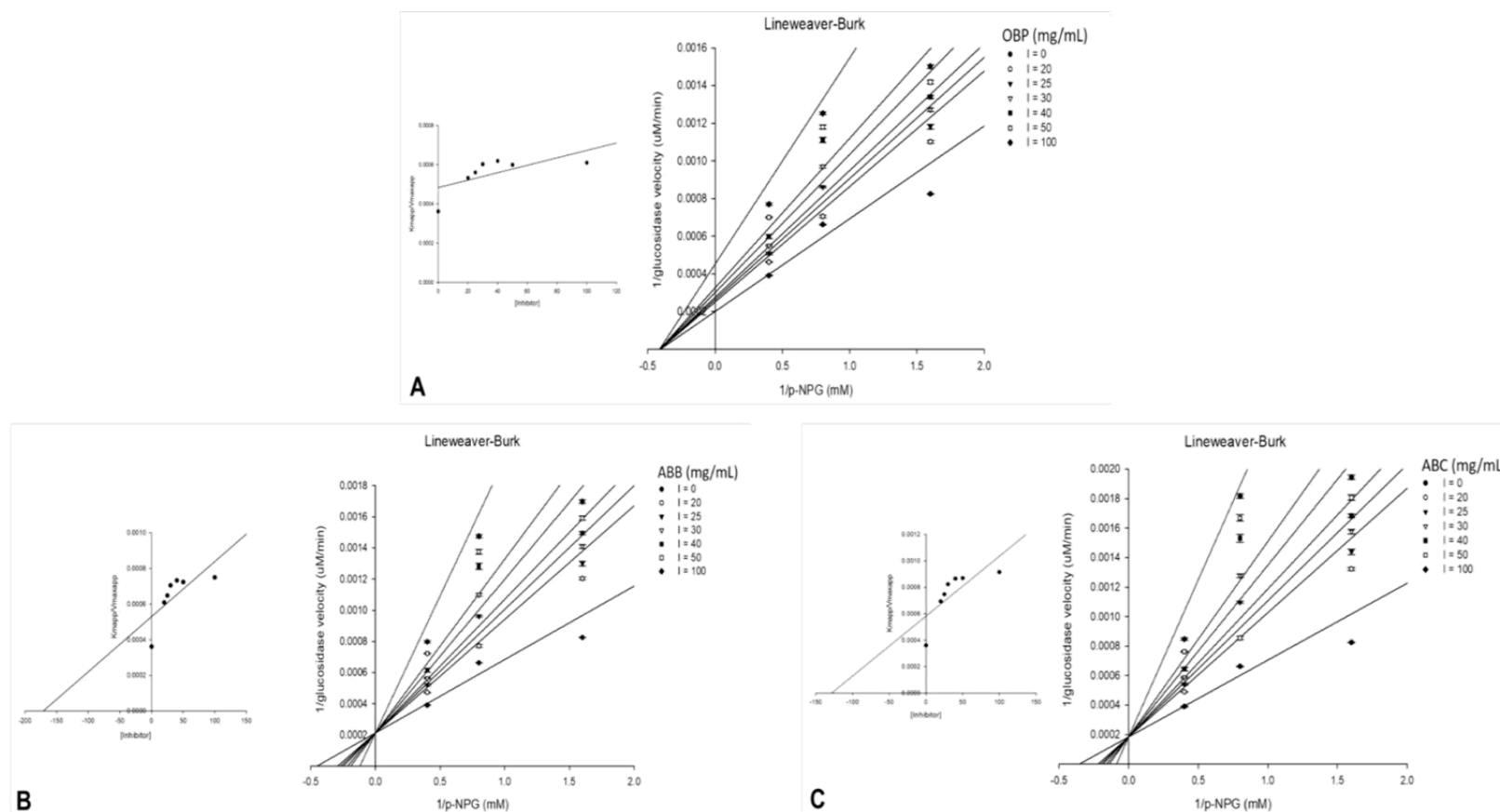


Figure 7.7 Lineweaver-Burk plot and direct linear plot (insets) for α -glucosidase inhibition by extracts of pastes

Inhibitors have illustrated in figures. Various concentrations of inhibitors are list in the legend entries. (A) OBP = oat bran paste; (B) ABB = 25% blueberry enriched oat bran paste; (C) ABC = 25% blackcurrant enriched oat bran paste.

Table 7-2 Detailed α -glucosidase inhibition kinetics constants of K_m^{app} and V_{max}^{app}

Groups	K_m^{app} (mg/mL)					V_{max}^{app} (mM/min)						
	A	B	C	D	E	A	B	C	D	E	F	G
Acarbose	1.471	1.590	2.039	2.812	2.272	4.067	3.888	3.971	4.068	3.278	2.991	
Oat bran	1.471	1.562	1.992	2.724	2.203	4.067	3.860	3.930	4.001	3.230	2.932	
Blueberry powder	1.471	2.085	2.731	1.912	2.373	4.067	3.840	3.986	2.881	2.798	1.879	
Blackcurrant powder	1.471	0.823	0.991	1.190	0.866	4.067	2.477	2.338	2.070	1.622	1.260	
Oat bran paste	1.471	2.134	1.967	1.957	1.766	4.067	4.007	3.513	3.249	2.853	2.175	1.903
25% blueberry enriched pastes	1.471	2.662	2.479	2.514	2.281	4.067	4.370	3.826	3.564	3.112	2.312	2.011
25% blackcurrant enriched pastes	1.471	3.285	3.090	3.205	2.919	4.067	4.729	4.132	3.890	3.369	2.415	2.082

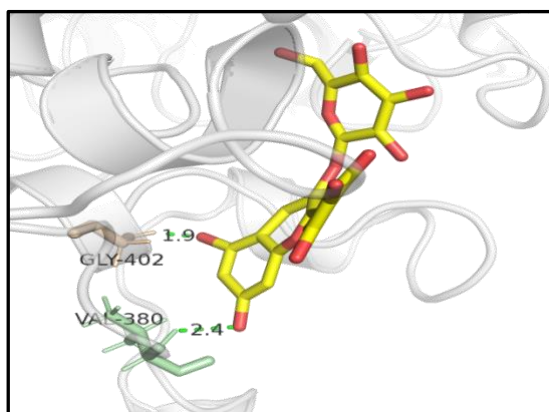
Data were taken from Lineaweaver-Burk plot. Characters (A to G) represent the concentrations of inhibitors (A = 0; E or G = the highest concentration; Increasing order from A to F or G).

7.3.5 Molecular docking study on α -glucosidase

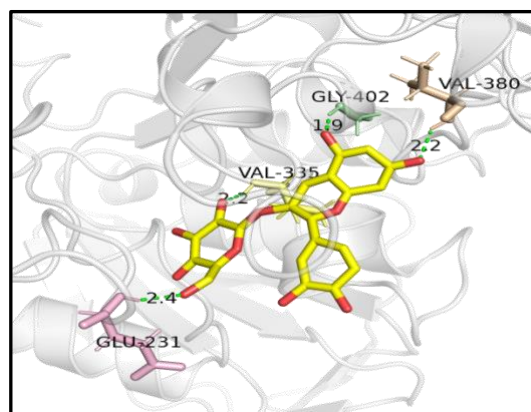
To provide a deeper insight into the interaction α -glucosidase with anthocyanins, a molecular docking study was performed. Four main anthocyanins inhibitors were docked into the catalytic sites of α -glucosidase. **Fig. 7-8** predicts the binding modes of the four main anthocyanins (del-3-glu, cya-3-glu, mal-3-glu, and cya-3-rut) with α -glucosidase. The model illustrates that the anthocyanins could fit into the binding pocket of the active site (**Table 7-3**). Del-3-glu exhibited the strongest binding affinity to α -glucosidase (-9.7 kcal/mol), followed by cya-3-glu. The number of hydroxyl groups in the B-ring of the four anthocyanidins were consistent with the results of the binding affinities. These results confirm that anthocyanins have the ability to enter into the active site of α -glucosidase and inhibit the catalytic action of α -glucosidase through hydrogen bonding. Thus, their significant α -glucosidase inhibitory effects were mainly due to the number of hydroxyl groups on the B-ring present in anthocyanins. When compared to the binding results of the four anthocyanidins with α -glucosidase, only cya-3-rut failed to bind to the side chain of GLY-402. Therefore, it can be hypothesised that the absence of GLY-402 residue might contribute to the disaccharide glucoside of cya-3-rut and further influence the binding affinity with α -glucosidase. This idea is consistent with a previous study (Ying Liu *et al.*, 2018), reporting that the addition of disaccharide affects the polarity of anthocyanins and different glycosides alter the chemical structure of anthocyanins, either of which may enhance or decrease the inhibition activity of anthocyanins. Interestingly, although cya-3-rut exhibited the weakest binding affinity with α -glucosidase, cya-3-rut accounted for the largest group of anthocyanins in the extract of 25% blackcurrant enriched paste. In terms of the α -glucosidase inhibitory activity, the extract of 25% blackcurrant enriched paste showed the strongest inhibitory effect compared to the other extracts of pastes.

Table 7-3 Predicted binding affinity of anthocyanins docked with α -glucosidase

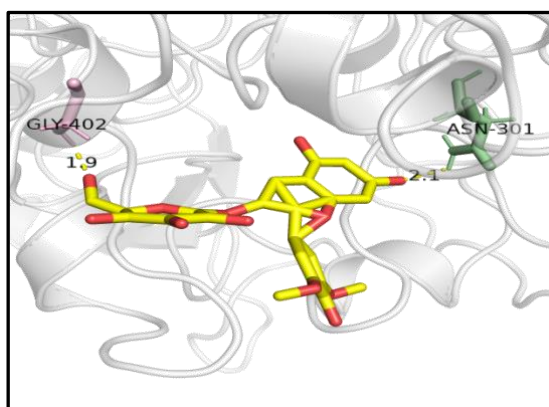
Affinity (kcal/mol)	No. of H-bonds	Amino acid residues
-9.7	2	GLY-402, and VAL-380
-9.3	4	GLU-231, VAL-335, GLY-402, and VAL-380
-8	2	GLY-402 and ASN-301
-7.6	4	ASN-174, TRP-130, ARG-158, ASP-132 and ASP-126



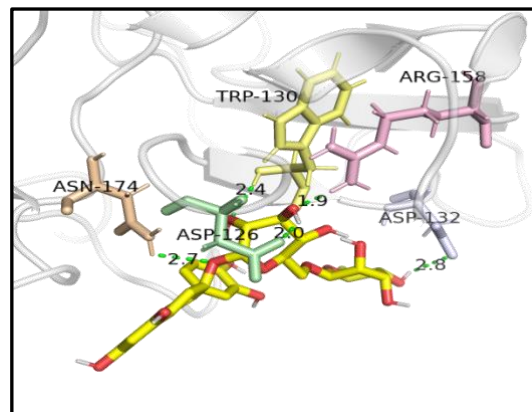
Del-3-glu



Cya-3-glu



Mal-3-glu



Cya-3-rut

Figure 7.8 Molecular docking studies on the interaction of the anthocyanins with α -glucosidase

7.4 Conclusion

This chapter studied the α -glucosidase inhibitory activity of the extracts from raw materials and blueberry and blackcurrant enriched pastes. The results showed that the extract from blackcurrant enriched pastes showed a stronger α -glucosidase inhibitory effect than the extract from the blueberry enriched pastes, which was opposite to the results of α -amylase inhibitory activity. According to the kinetic analysis of the enzymes, extracts from blueberry and blackcurrant enriched paste competitively inhibited α -glucosidase activity. These results also confirmed that the efficiency of α -glucosidase inhibition derived from the extracts of blueberry and blackcurrant enriched oat bran paste is dependent upon their site of action, their mechanism, and their binding affinities. Synergies occurring in the food matrix may enhance their inhibitory activity. Therefore, the inhibitory activity of the berry-derived polyphenols towards α -glucosidase gives the potential for T2DM patients to manage their own glycaemic control via dietary means. Once the “best” inhibitors are identified, they could be purified and formulated into drug-like treatments. However, these results were from *in vitro* studies, the inhibitory effects of the products on α -glucosidase should be investigated further via *in vivo* study to give a better understanding of the activity of the bioactive compounds.

Chapter 8

The combination of oat bran with blueberry or blackcurrant powder induces the apoptosis and inhibit the proliferation of HepG2 cell line

Abstract

Cereal foods supplemented with fruit ingredients have attracted the attention of consumers due to their rich phenolic contents and health promoting effects. In this study, the extract from oat bran enriched with 25% (w/w) of blueberry and blackcurrant powder, respectively, were used to investigate the food matrix effect on anticancer bioactivity towards HepG2 cell line using an *in vitro* digestion system. Results showed that the extract from the 25% of blueberry powder and blackcurrant powder enriched oat bran paste significantly inhibited the cell growth and cell invasion of HepG2 cell line. After experiencing the intestinal digestion, the anticancer activities of the extracts from these pastes decreased. Mechanistic studies suggested that the extract of oat bran paste containing blueberry or blackcurrant induced HepG2 cell apoptosis, which was initiated through cell cycle arrest and regulation of the expression of apoptotic-related proteins, including Bcl-2, Bax and caspase-3. The intracellular ROS level of treated-HepG2 cell line increased via regulation of the nuclear factor-like 2 (Nrf2)/heme oxygenase 1 (HO1) signalling pathway. These findings revealed the anti-cancer potential of the extract from the oat bran paste enriched with blueberry or blackcurrant powders towards HepG2 cell lines.

Keywords: digestion; ROS; apoptosis; proliferation; cell cycle; Bcl-2; Bax; caspase-3

8.1 Introduction

Hepatocellular carcinoma (HCC) is considered to be the most common primary cancer of the liver and it is also the fastest growing cause of cancer-related death in the US (Bray *et al.*, 2018), the second leading cause of cancer death in East Asia, and the sixth most common cancer in western countries (Ferlay *et al.*, 2019). The drastic rise in the incidence of liver cancer, lack of adequate treatment, and the serious side effects of the existing medicines make it urgent to look for alternative and novel treatments. Natural therapeutics are being screened for cancer prevention and alternative treatment due to their low toxicity. Natural flavonoids have attracted the attention of scientists as they have been found to have the minimum toxicity. Flavonoids are also considered as potential anticancer agents (Chabot, Touil, Pham, & Dauzonne, 2010). Blueberries and blackcurrants are rich in flavonoids, and several studies have demonstrated that blueberries and blackcurrants, as well as their active constituents, show promising outcomes as anticancer agents (Gopalan *et al.*, 2012; Ma *et al.*, 2018; Pan, Liu, Liu, & Wang, 2019; Wang, Liu, Xu, & Liu, 2017). Oat bran is normally consumed as a ready-to-eat breakfast and porridge. The interest in consuming oat bran has been mainly due to its macronutrient compositions such as fibre and high β -glucan content. Evidence has shown that oat bran also contains phenolic compounds, which in combination with fibre exert a protective effect against cancer (Turrini, Maffei, Milelli, Calcabrini, & Fimognari, 2019). However, no study is available on the influence of supplemental blueberry and blackcurrant regarding its anticancer activities on oat bran. In addition, there are few, if any, studies which focus on the effects of the human digestion system on the anticancer activities of investigated foods, specifically performing on HepG2 cell line. Therefore, *in vitro* digestion was used in this study to evaluate the anticancer activity of undigested and digested oat bran enriched with blueberry powder or blackcurrant powder. The potential anticancer mechanisms were also

investigated.

8.2 Methods

8.2.1 Preparation of oat bran paste

Pastes were prepared as described in 3.2.

8.2.2 Simulation of the *in vitro* digestion

The *in vitro* digestion is described in 3.6.

8.2.3 Extraction of raw materials and pastes

The extraction of both undigested and digested samples was carried out as described in 3.5.

8.2.4 Cell viability of HepG2 cells

The cell viability was analysed using CC-8 kit as described in 3.16.1.

8.2.5 Induction of cell apoptosis of HepG2 cell line via flow cytometry assay (FCA)

The cell apoptosis of HepG2 cell line was tested as described in 3.16.2.

8.2.6 Hoechst staining

The Hoechst staining was performed to observe the cell apoptosis using microscope as described in 3.16.3.

8.2.7 Inhibition of cell invasion of HepG2

The cell invasion of HepG2 cell line was performed as described in 3.16.4.

8.2.8 Cell cycle of HepG2 cells analysis

The cell cycle of HepG2 cell line was analysed to study the extracts for inhibition of cell proliferation, as described in 3.16.5.

8.2.9 EdU staining

The EdU staining was employed to observe the cell proliferation of HepG2 cell line as described in 3.16.6.

8.2.10 Induction of intracellular ROS generation of HepG2

The ROS generation of HepG2 cell line was detected via FCA as described in 3.16.7.

8.2.11 Western blotting assay

The Western blotting assay was used to observe the protein expression of Bax, Bcl-2, Caspase-3, Nrf2, and HO1, as described in 3.16.8.

8.2.12 Real-time quantitative PCR assay (RT-PCR)

The RT-PCR assay was used to observe the protein expression of Nrf2 and HO1, as described in 3.16.9.

8.2.13 Statistical analysis

The results were analysed as described in 3.17.

8.3 Results and discussion

8.3.1 The extracts of blueberry and blackcurrant enriched pastes suppressed the HepG2 cell growth

A CCK-8 assay was performed to evaluate the effects of the extracts from blueberry and blackcurrant enriched pastes on HepG2 cell viability. As shown in **Fig. 8-1a & 8-1b**, both undigested and digested extracts inhibited HepG2 cell growth in dose-dependent manners. Both undigested and digested extracts of 25% blueberry and 25% blackcurrant enriched pastes exhibited stronger inhibitory activity against cell growth of HepG2, compared to the extract of oat bran paste ($p < 0.01$). **Fig. 8-1c** presents the IC₅₀ value of all extracts against cell growth. Extracts of undigested 25% blackcurrant enriched pastes showed the lowest IC₅₀ value of

133.35 µg/mL. After digestion, all of the extracts had an increased IC₅₀ value, which indicated that their ability to inhibit HepG2 cell growth was reduced. However, the extracts of digested 25% blueberry enriched paste (252.42 µg/mL) and 25% blackcurrant enriched paste (182.16 µg/mL) still presented the lower IC₅₀ compared with the extract of digested oat bran paste (449.71 µg/mL) ($p < 0.01$). A preliminary study showed that these extracts had no inhibitory activity on normal liver cell growth until the concentrations were 4,000 µg/mL. This cell line specific inhibitory efficacy could be responsible for the different biochemical characteristics between normal and Hela cells (Lu, Shen, Yang, & Gu, 2016). The different biological activities of these two cell lines, normal and Hela cells, could be due to the accumulation of glycosylated flavanols in the cell medium or to a different sensitivity of the cell lines to the phenolic compounds (Cianciosi *et al.*, 2018).

According to literature, this is the first report investigating the anticancer properties of extracts of blueberry and blackcurrant enriched pastes against HepG2 cell line and the influence of the *in vitro* digestion. Some studies have reported the anticancer properties of anthocyanidin-rich extracts against a Caco-2 cell model. They also reported that after *in vitro* digestion, the bioactivity of these extracts decreased due to the degradation of anthocyanidins (Kamiloglu *et al.*, 2015; Kubow *et al.*, 2017). These results are similar to the observations in this chapter, and the previous chapters. Even though purification and isolation of the most effective bioactive compounds from the blueberry and blackcurrant enriched pastes was not performed in this study, it can be assumed that the bioactivity of the extracts against the HepG2 cell line may be due to the presence of the anthocyanidins in the blueberry and blackcurrant enriched pastes. The bioactivity of phenolic compounds is mainly determined by their bioaccessibility in the gastrointestinal tract, and their cellular absorption and internal transformation (Aragonès, Danesi, Del Rio, & Mena, 2017). This chapter reports on the extraction of amounts of bioaccessible phenolic compounds from blueberry and

blackcurrant enriched pastes after the *in vitro* gastrointestinal digestion. Results showed that the digestion process decreased the phenolic compound content to some extent. Nevertheless, it could be interesting to underline how the sensitivity to the presence of phenolic-rich extracts increased after *in vitro* digestion.

8.3.2 The extracts from the blueberry and blackcurrant enriched pastes induced the HepG2 cell apoptosis

According to the IC₅₀ value of all the extracts against HepG2 cell growth, a level of 450 µg/mL was chosen as the treating concentration to detect apoptosis of HepG2 cells by using Annexin V/PI staining method. As shown in **Fig. 8-2**, stimulation with the extracts of 25% blueberry and 25% blackcurrant enriched pastes significantly increased the number of apoptotic HepG2 cells, compared with oat bran paste and the control group ($p < 0.01$). Furthermore, the ability of the undigested extracts to induce the HepG2 cell apoptosis was stronger than the digested extracts ($p < 0.01$). Extracts of digested 25% blackcurrant enriched paste showed the strongest ability to induce the HepG2 cell apoptosis than the other digested extracts ($p < 0.01$). Fluorescence microscopy analysis via Hoechst staining showed clear morphological changes in the nucleolus of HepG2 cell line (**Fig. 8-3**). Hoechst staining indicated that the marked fragmented nuclei of HepG2 cells stimulated with the extracts from 25% berry powder enriched pastes were significantly reduced and shrunk.

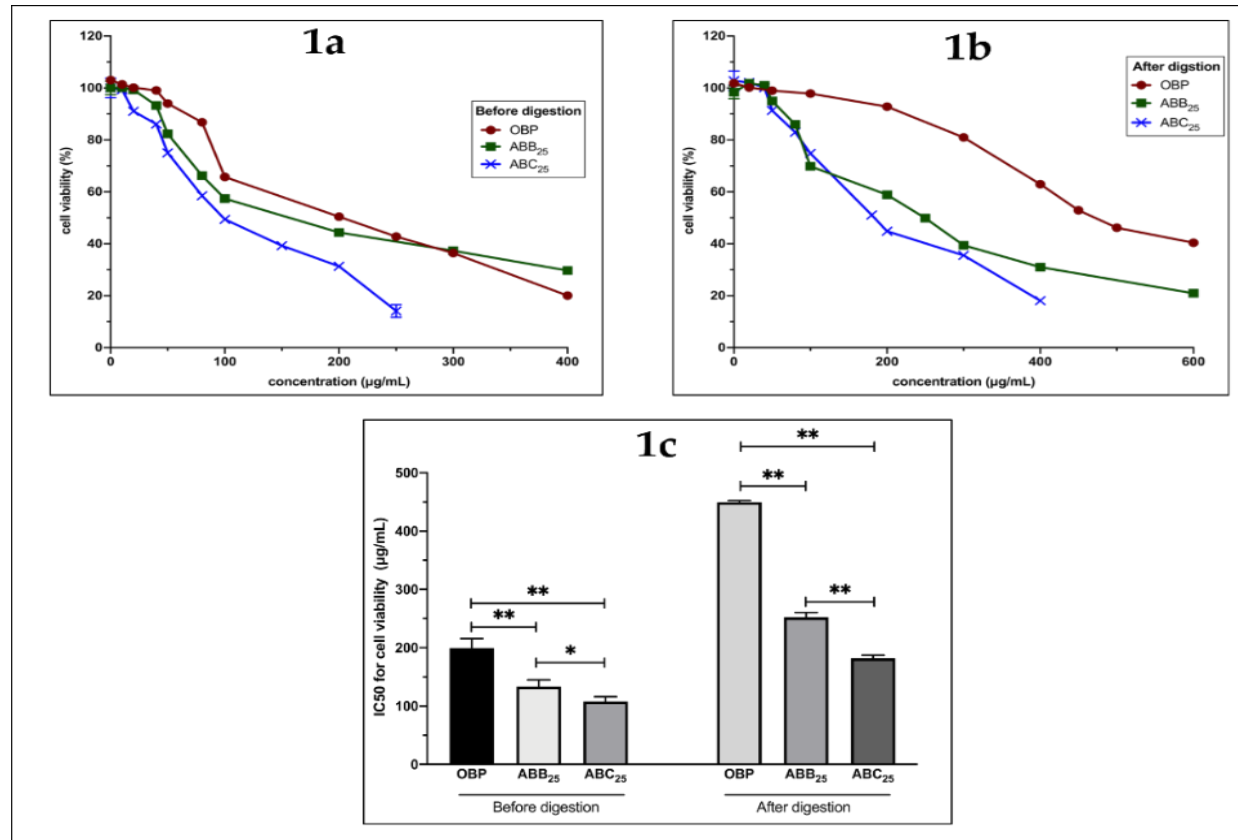


Figure 8.1 Inhibition of HepG2 cell growth by extracts of pastes.

HepG2 cell viability after treating with the extracts of undigested (1a) and digested pastes (1b) for 24 h; IC₅₀ value (1c) for HepG2 cell growth. Values = mean ± SD, n = 3 (**p* < 0.05, ***p* < 0.01 compared to the control group). OBP = pure oat bran paste; ABB₂₅ = 25% blueberry enriched paste; ABC₂₅ = 25% blackcurrant enriched paste

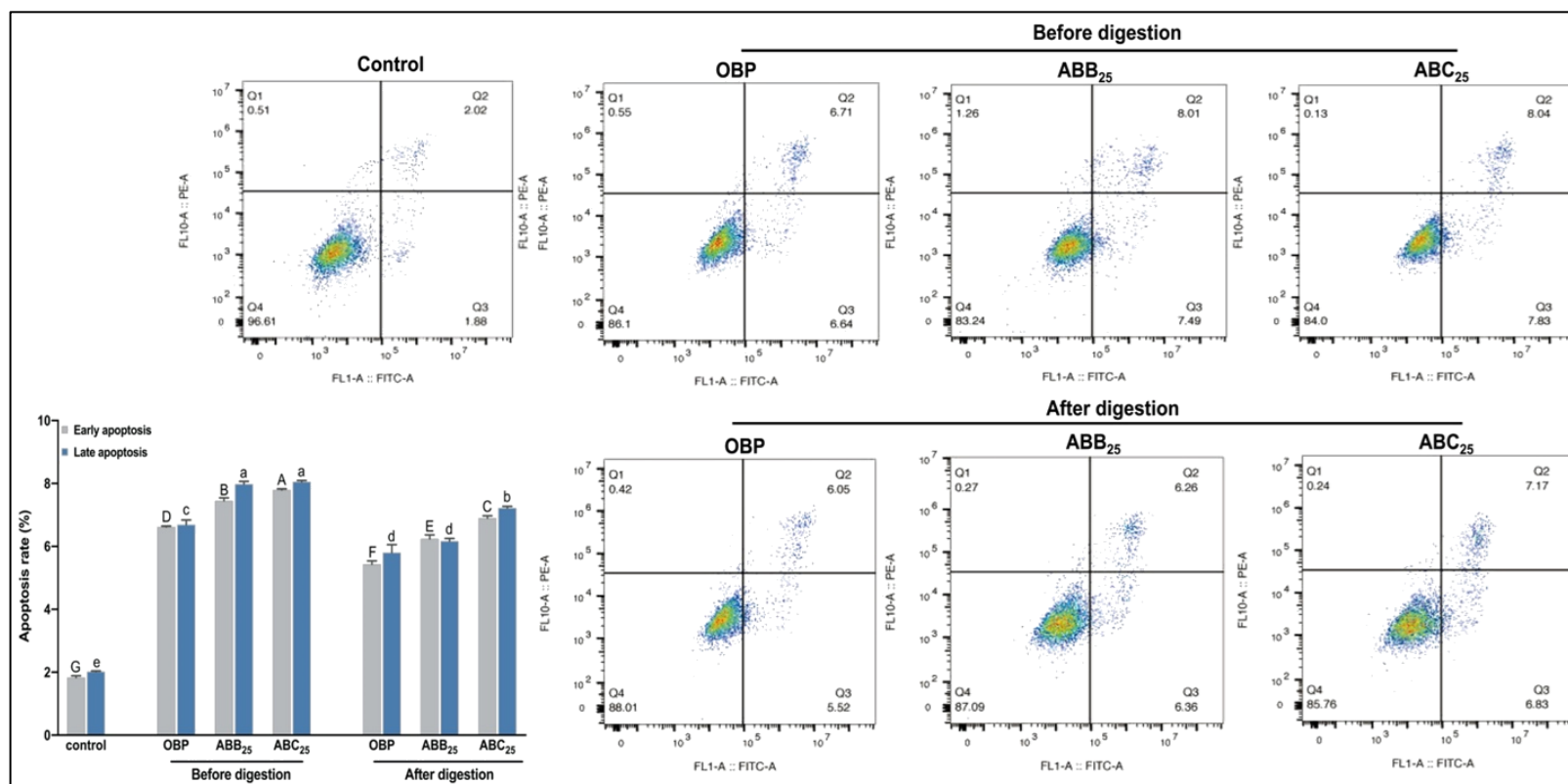


Figure 8.2 Induction of HepG2 cell apoptosis by extracts of pastes

Values = mean \pm SD, $n = 3$. Values with different upper-case letters are significantly different within the early apoptosis, while values with lower case letters are significantly different within the late apoptosis ($p < 0.05$). OBP = oat bran paste; ABB₂₅ = 25% blueberry enriched paste; ABC₂₅ = 25% blackcurrant enriched paste.

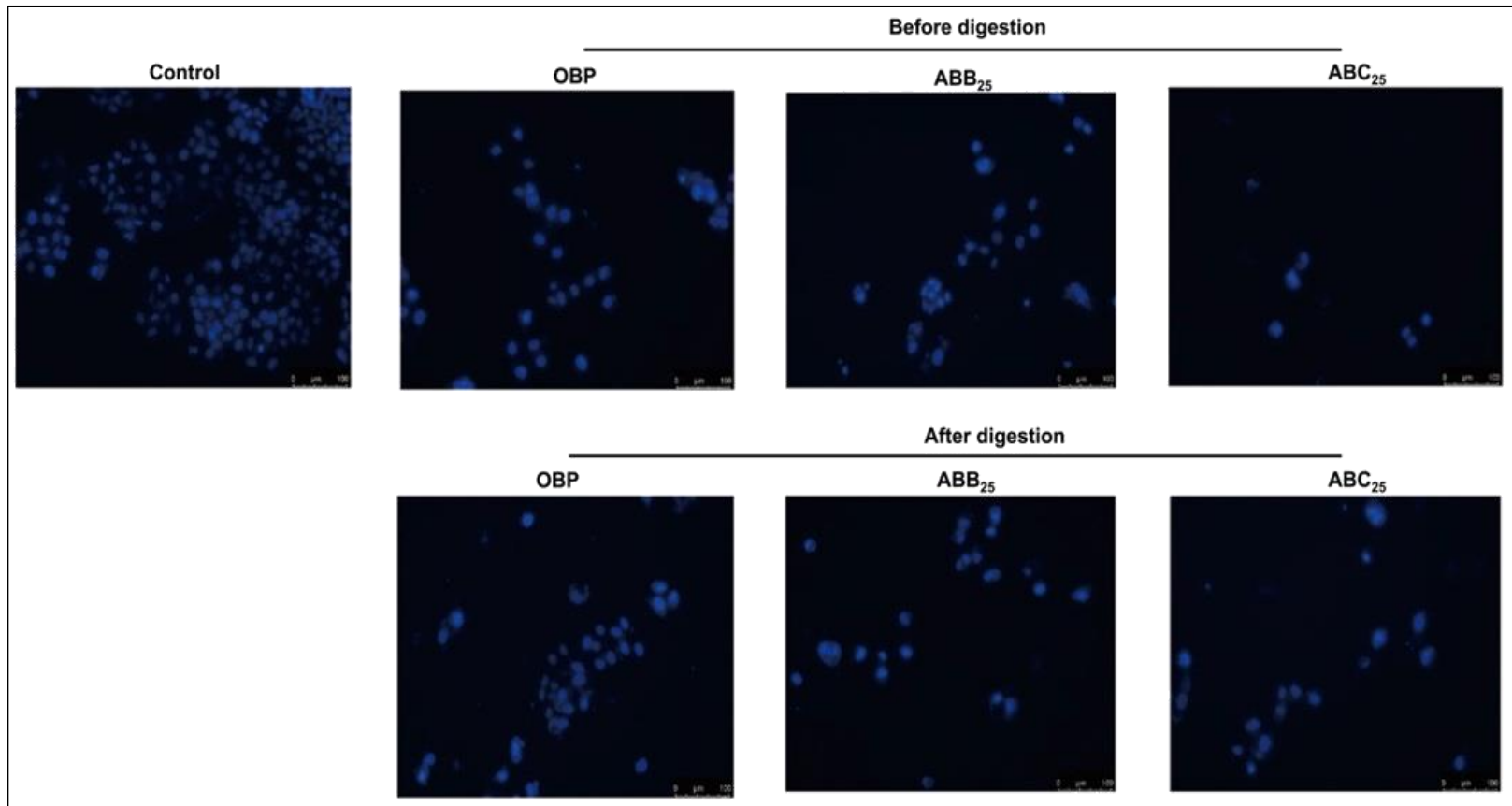


Figure 8.3 Hoechst staining of apoptotic HepG2 cells

OBP = oat bran paste; ABB₂₅ = 25% blueberry enriched paste; ABC₂₅ = 25% blackcurrant enriched paste.

8.3.3 Induction of HepG2 cell apoptosis through Bcl-2/caspase signalling pathway

To further identify the potential mechanisms involved in the induction of apoptosis, the IC₅₀ and half of IC₅₀ concentration for extracts of digested blueberry (250 µg/mL and 125 µg/mL) and blackcurrant enriched pastes (180 µg/mL and 90 µg/mL) treated HepG2 cells were used to observe the expression of apoptosis-regulating proteins. **Fig. 8-4** shows that the expression of Bcl-2/Bax was significantly decreased in a dose-dependent manner following treatment with extracts of digested 25% blueberry and 25% blackcurrant enriched pastes. In addition, we examined the expressions levels of caspase-3. The result suggest that the extracts of blueberry and blackcurrant enriched pastes induced HepG2 cell apoptosis possibly through the activation of caspase-mediated pathways.

The Bcl-2 family proteins are well-known as one of the main factors involved in the apoptotic pathway as they control apoptosis. Bcl-2 is a strong anti-apoptotic protein, while Bax is an apoptosis inducer (Warren, Wong-Brown, & Bowden, 2019). Caspase-3 plays an important role as a downstream effector of apoptosis and the increased caspase-3 level is always used as an indicator of apoptosis (Ponder & Boise, 2019). The results in this chapter reveal that the extracts of digested 25% blueberry and 25% blackcurrant enriched pastes activated caspase-3 in the HepG2 cell line in a concentration-dependent manner. The potential mechanisms involved in the mitochondrial apoptotic pathway in 25% blueberry and 25% blackcurrant enriched pastes induced cell apoptosis were observed in Bcl-2 and Bax expression levels. Protein expression analysis showed that the extracts of blueberry and blackcurrant enriched pastes downregulated the Bcl-2 expression and upregulated the Bax expression, consequently promoting the cell apoptosis.

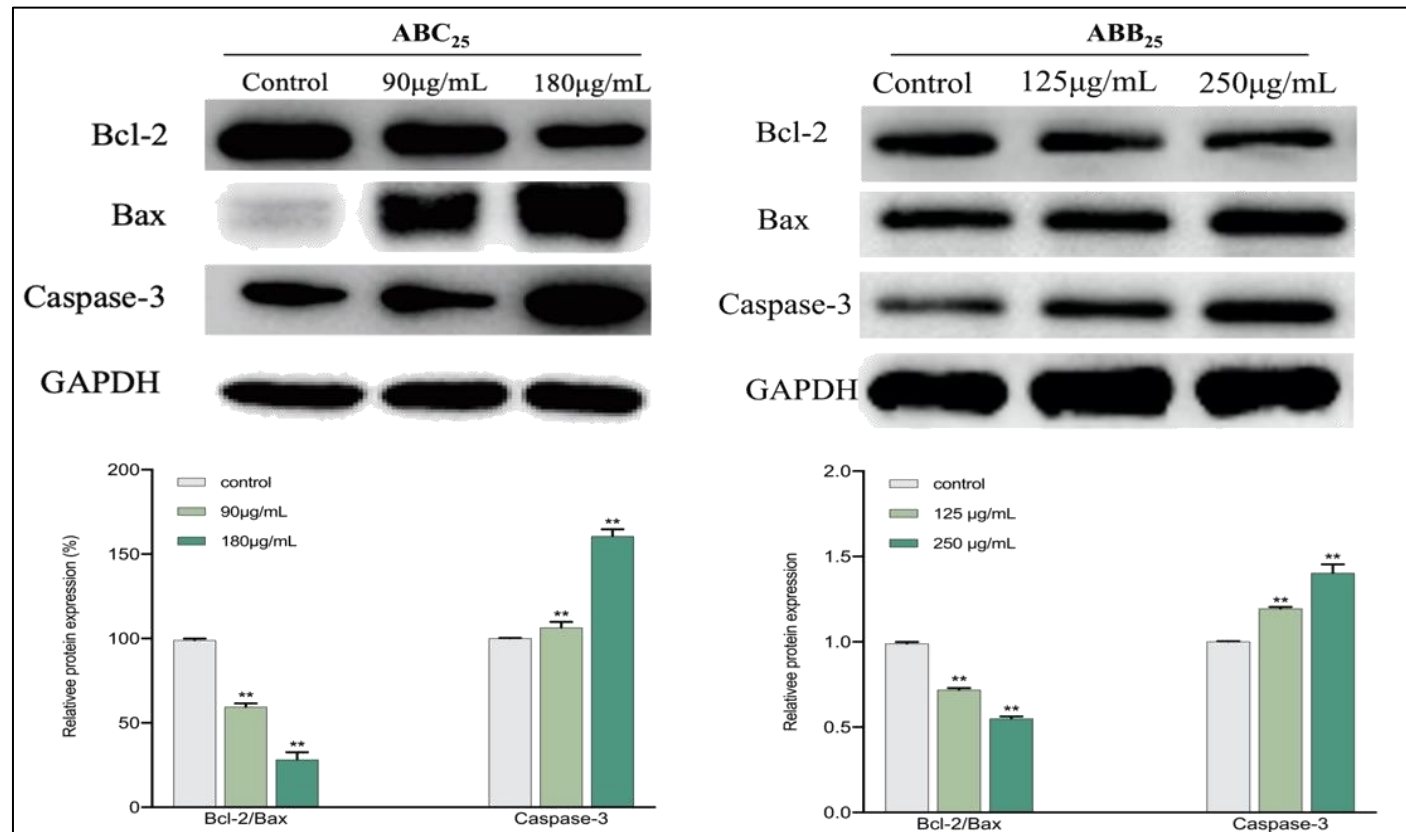


Figure 8.4 The expression of Bcl-2, Bax and caspase-3 by western blotting.

Extracts of digested blueberry and blackcurrant enriched pastes induced HepG2 cell apoptosis via regulation the expression of Bcl-2/Bax and caspase-3 protein levels. Values = the mean \pm SD, $n = 3$ (* $p < 0.05$, ** $p < 0.01$ compared to the control group). OBP = oat bran paste; ABB₂₅ = 25% blueberry enriched paste; ABC₂₅ = 25% blackcurrant enriched paste.

8.3.4 The extracts from blueberry and blackcurrant enriched pastes inhibited the HepG2 cell invasion

As shown in **Fig. 8-5**, treatment with undigested and digested extracts from 25% blueberry and 25% blackcurrant enriched pastes for 24 h significantly decreased the number of invading HepG2 cells compared with oat bran paste ($p < 0.01$). The extract of 25% blackcurrant enriched paste exhibited the highest invasive ability than the other extracts of pastes. Cell invasion is the key character of cancer progression. Therefore, suppression of the cell invasion may ensure a relatively longer period of survival for patients (Ponder & Boise, 2019). The potential action of the extracts from blueberry and blackcurrant enriched pastes against HepG2 cell invasion indicated that they might be an efficient treatment in preventing cancer progression and should be further investigated via *in vivo* studies.

It is worth noting that after *in vitro* digestion, the ability of the extracts to inhibit the HepG2 cell invasion was lower than the undigested extracts. Several factors could explain this phenomenon. First of all, the neutral pH conditions in cellular mediums may affect the activity of the bioactive ingredients. A preliminary study conducted during the research showed that during the intestinal digestion, the antioxidant capacity of phenolics reduced, which was related to the alkaline pH condition since these phenolic compounds are very sensitive to the alkaline pH conditions (Kumarappan, Thilagam, & Mandal, 2012). Herein, the reduced anthocyanin contents in the digested extracts (compared to the undigested extracts) may be responsible for the reduction of the ability to inhibit the HepG2 cell invasion (Vulić *et al.*, 2019; Zargoosh, Ghavam, Bacchetta, & Tavili, 2019).

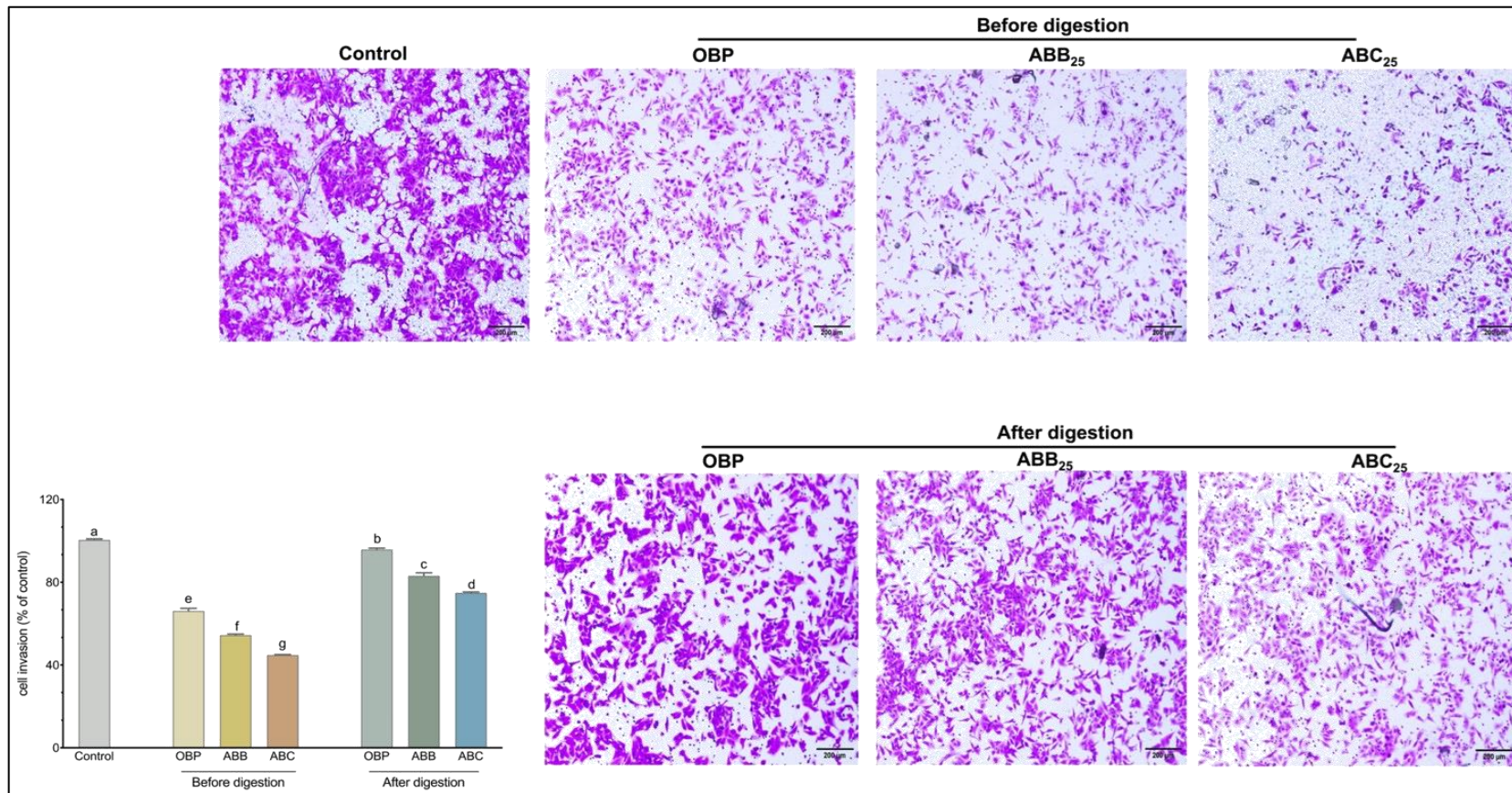


Figure 8.5 Inhibition of the HepG2 cell invasion by the extracts of paste.

Values = mean \pm SD, n = 3. Values with different letters are significantly different ($p < 0.05$). OBP = oat bran paste; ABB₂₅ = 25% blueberry enriched paste; ABC₂₅ = 25% blackcurrant enriched paste

8.3.5 The extracts from blueberry and blackcurrant enriched pastes inhibited the HepG2 cell proliferation

As shown in **Table 8-1**, the analysis of the cell cycle revealed that the extracts significantly increased the percentage of HepG2 cells in G1 phase (growth) and decreased the percentage of cells in the S phase (DNA synthesis), compared with the untreated control group ($p < 0.01$), indicating that the extracts of pastes induced arrest cell cycle at the G1-S checkpoint (growth to DNA synthesis) (**Fig. 8-6**). The same trend presented in cell growth and apoptosis of HepG2, the extract of 25% blackcurrant enriched paste showed the highest arrest ability compared to the other extracts, while the digested extracts inhibited fewer cells to transit to the S phase. These results suggest that apoptotic cell death was caused by disrupting specific phase during the HepG2 cell cycle.

EdU staining (**Fig. 8-7**) was used to quantify HepG2 cell proliferation, while Hoechst staining was used to observe the nucleolus of treated-HepG2 cells. Compared to the control group, treatment with the extract of oat bran paste, 25% blueberry and 25% blackcurrant enriched pastes reduced the number of EdU positive HepG2 cells. The extract of 25% blackcurrant enriched paste show the most repression of the HepG2 cell proliferation, as evidenced by the decreased ratio of relative proliferation HepG2 cells ($p < 0.01$) (**Fig. 8-8**). Several anticancer agents result in cell cycle arrest and are clinically effective for the cancer treatments, and drugs with apoptosis-inducing properties always reduce the potential of drug resistance (Bayat Mokhtari *et al.*, 2017; Zhang *et al.*, 2017; Zhang *et al.*, 2020). These results show that cell proliferation was negatively regulated by blueberry and blackcurrant enriched pastes, and their antiproliferative ability could be augmented via arresting the HepG2 cell cycle.

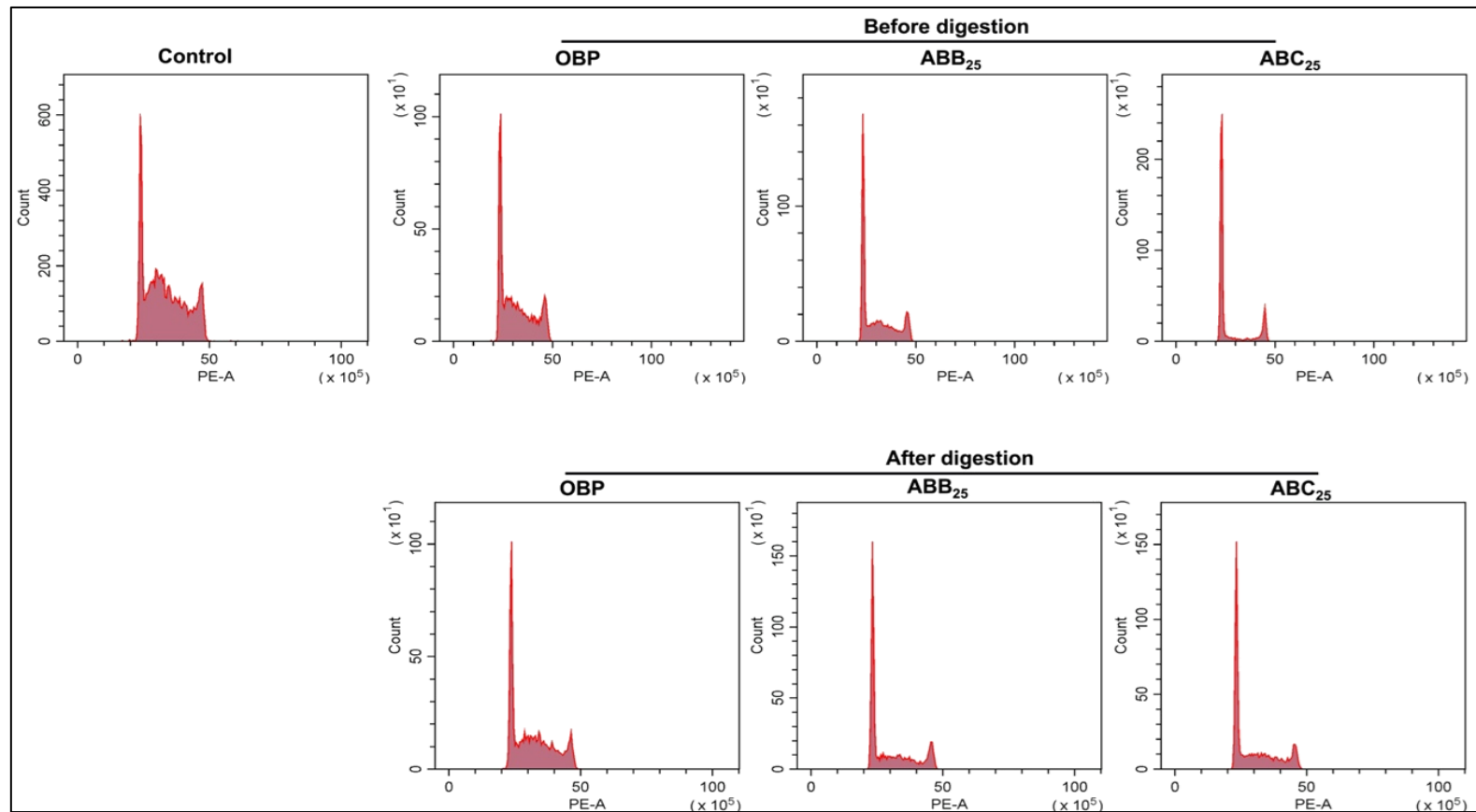


Figure 8.6 Inhibition of the HepG2 cell cycle by the extracts of pastes

OBP = oat bran paste; ABB₂₅ = 25% blueberry enriched paste; ABC₂₅ = 25% blackcurrant enriched paste

Table 8-1 The relative cell number at different phases of HepG2 cell cycle

Group	G1/G0	S	G2/M
Control	29.43 ± 0.56 ^e	66.47 ± 0.46 ^a	4.09 ± 0.27 ^c
Extracts from gastric phase			
Oat bran paste	48.97 ± 1.83 ^{bc}	45.07 ± 1.05 ^d	5.96 ± 0.78 ^b
25% blueberry enriched paste	51.77 ± 1.31 ^b	42.24 ± 0.64 ^e	5.98 ± 0.69 ^b
25% blackcurrant enriched paste	68.30 ± 0.56 ^a	26.11 ± 0.54 ^f	5.59 ± 0.20 ^b
Extracts from intestinal phase			
Oat bran paste	33.74 ± 0.45 ^d	62.80 ± 0.14 ^b	3.46 ± 0.32 ^c
25% blueberry enriched paste	42.50 ± 1.32 ^c	49.38 ± 0.52 ^c	8.10 ± 0.80 ^a
25% blackcurrant enriched paste	44.61 ± 1.32 ^c	50.76 ± 0.86 ^c	4.63 ± 0.56 ^b

Values = mean ± SD, n = 3. Values in the same column with different letters are statistically different ($p < 0.05$). G0 = rest; G1 = growth; S = DNA synthesis; G2 = growth and preparation for mitosis; M = mitosis (cell division).

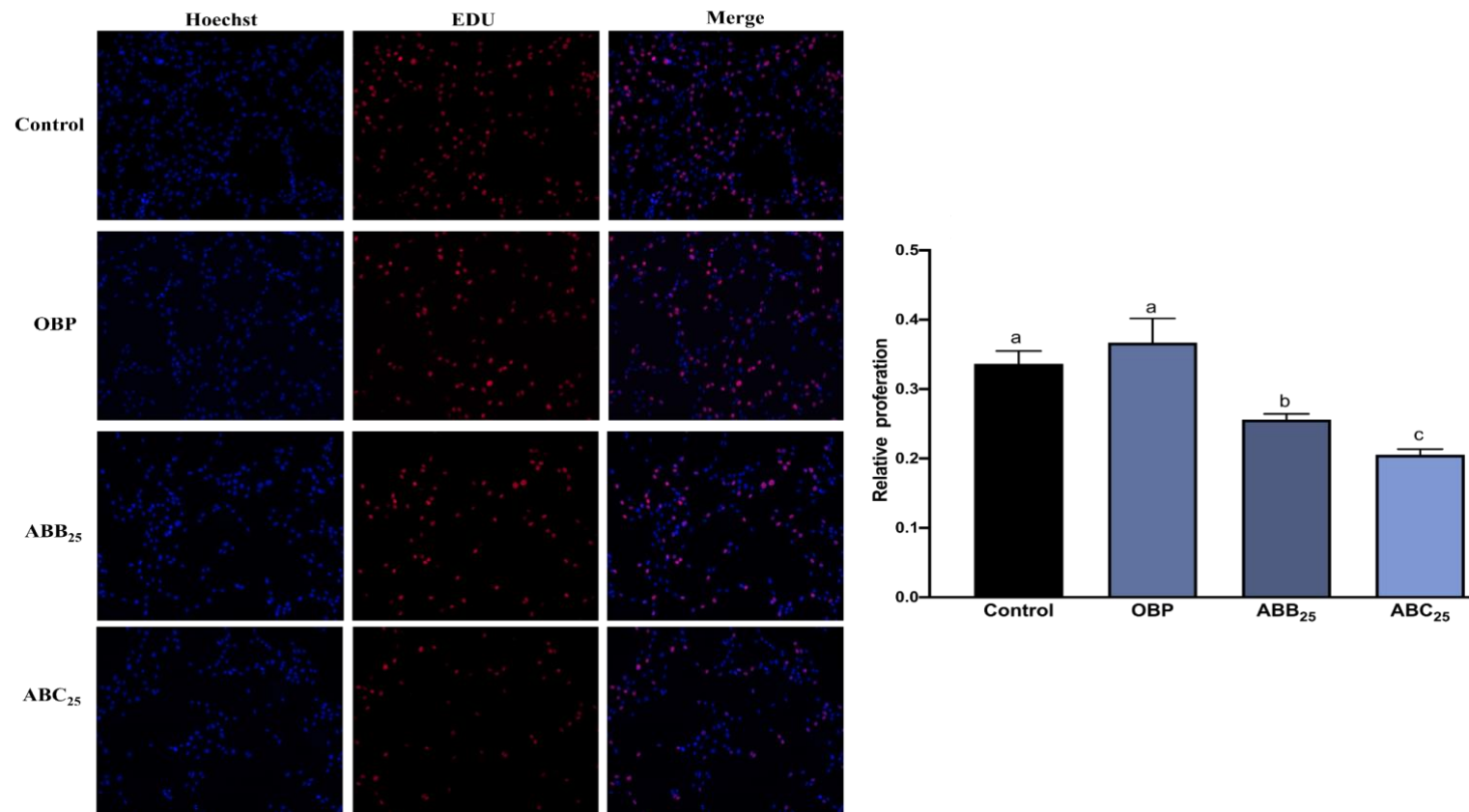


Figure 8.7 The relative proliferation ratio of HepG2 cells by EdU staining

Values = mean ± SD, n = 3. Values with different letters present the significant difference (p < 0.05). OBP = oat bran paste; ABB₂₅ = 25% blueberry enriched paste; ABC₂₅ = 25% blackcurrant enriched paste.

8.3.6 Extracts of blueberry and blackcurrant enriched pastes induced reactive oxygen species (ROS) overproduction

The intracellular ROS generation levels in HepG2 are presented in **Fig. 8-8**. Stimulation with the extracts of 25% blueberry and 25% blackcurrant enriched pastes significantly increased the intracellular ROS production of HepG2 cells, when compared to stimulating with the extract of oat bran paste ($p < 0.01$). The extract from 25% blackcurrant enriched paste induced the largest amount of ROS production of HepG2 cell than the other extracts ($p < 0.001$). It was observed that stimulation with the extracts from undigested pastes considerably augmented the ROS levels when compared to stimulating with the extracts from digested pastes ($p < 0.01$). Western blotting and RT-PCR assays were performed to investigate the effects of extracts from the digested 25% blueberry and blackcurrant enriched pastes on the nuclear factor erythroid 2-related factor 2 (Nrf2) expression level of HepG2 cells. As shown in **Fig. 8-9**, the extracts of digested blueberry and blackcurrant enriched pastes significantly inhibited the Nrf2 signalling pathway by down-regulation of the mRNA and protein expressions of Nrf2 as well as its downstream factor heme oxygenase 1 (HO1).

ROS participates in the mechanisms of numerous anticancer drugs via activating apoptotic signalling pathways during chemotherapy (Perillo *et al.*, 2020). It has been suggested that excess ROS may cause DNA damage and activate the Nrf2 and downstream factors, such as HO1 (Park *et al.*, 2019). Several studies have demonstrated the critical role of ROS in the signalling pathways induced by anticancer drugs (Zaidieh, Smith, Ball, & An, 2019). The results from this chapter revealed that ROS play a critical role in cell apoptosis, and the Nrf2 pathway may be involved in HepG2 cell apoptosis induced by the extracts of 25% blueberry and 25% blackcurrant enriched pastes. Similar studies have also illustrated that digestion significantly reduced the antioxidant activity of phenolic compounds via induction of ROS levels (Aggarwal *et al.*, 2019; Mao *et al.*, 2020; Meier *et al.*, 2017). Previous studies utilised the Caco-2/HepG2

co-culture system to study the effects of the digestion on the bioavailability of phenolic compounds and revealed that the reduction of their bioavailability may be correlated to the poor Caco-2 cellular transport and the complicated biotransformation of the microbial phenolic metabolites (Borges, Pereira, Cabrera–Vique, & Seiquer, 2017; Garbetta *et al.*, 2018).

8.4 Conclusion

In conclusion, extracts from oat bran paste enriched with 25% blueberry and blackcurrant powder, were investigated against the HepG2 cell line for their potential anticancer activity. The results indicated that enrichment of oat bran with 25% blueberry powder or blackcurrant powder induced a significant anticancer activity against the HepG2 cell line compared with consuming oat bran paste only. Enrichment with 25% blackcurrant exhibited a stronger anticancer ability than the enrichment with 25% blueberry. The anticancer effect was not as strong after digestion due mainly to the variation of polyphenols. The key driving mechanisms for their anticancer activities are considered to be the induction of ROS mitochondrial apoptosis via Nrf2/HO1 signalling pathway and apoptotic induction via regulation the expression of B2/Bax and caspase-3. Hence, for the future work, a combination of the *in vitro* digestion system with the Caco-2/HepG2 co-culture system should be developed to focus on the production of the metabolites of polyphenols that are predominant in the circulation. Further studies are necessary to confirm the proposed pathways of metabolism of these extracts during the incubation with cell lines, with purification of the most effective phenolic compounds to investigate their potential role in anticancer activity.

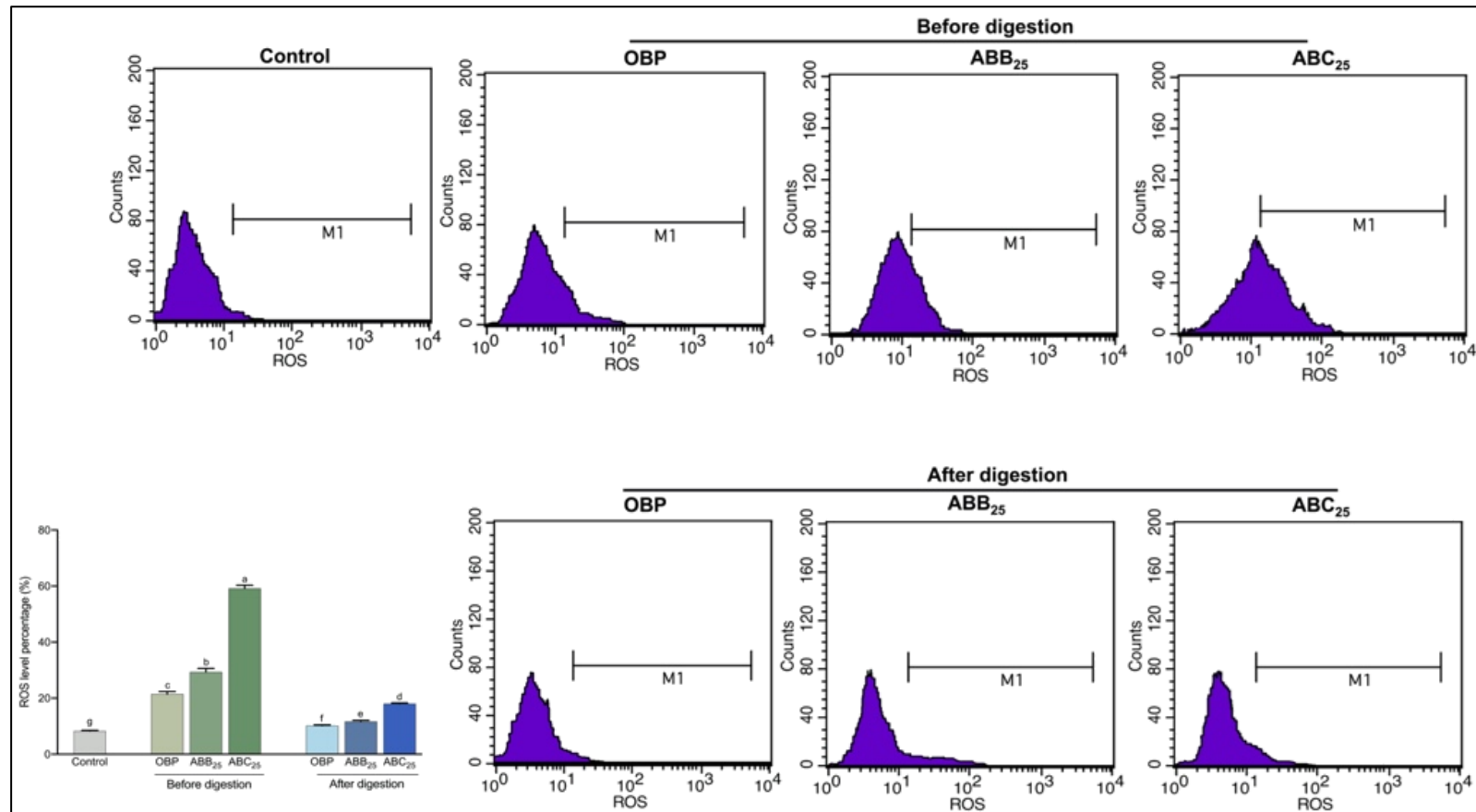


Figure 8.8 Induction of the Intracellular ROS generation by extracts of pastes.

Values = mean \pm SD, $n = 3$. Values with different letters are significantly different ($p < 0.05$). OBP = oat bran paste; ABB₂₅ = 25% blueberry enriched paste; ABC₂₅ = 25% blackcurrant enriched paste.

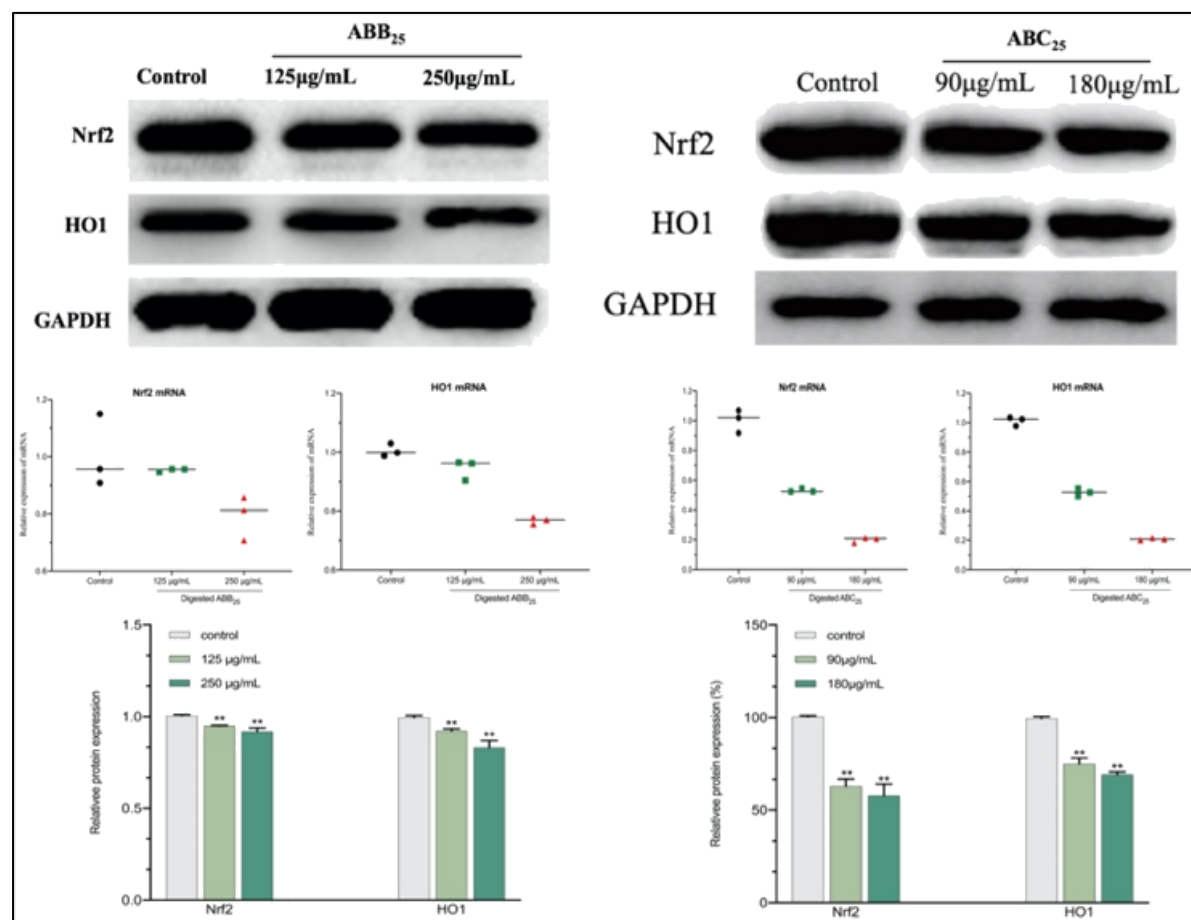


Figure 8.9 The expression of Nrf2 and HO1 by western blotting and RT-PCR.

Values = mean \pm SD, $n = 3$. Values with different letters are significantly different ($p < 0.05$). OBP = oat bran paste; ABB₂₅ = 25% blueberry enriched paste; ABC₂₅ = 25% blackcurrant enriched paste.

Chapter 9

General conclusion and future work

9.1 Aims and hypotheses

Oat bran pastes enriched with blueberry powder or blackcurrant powder were prepared to investigate the interaction of the bioactive ingredients from blueberry and blackcurrant powder with the other food components, such as protein, lipids, and fibre, present in the food matrix. The pasting properties, proximal composition, *in vitro* starch digestion, antioxidant capacity, and digestive enzyme inhibitory activities of the oat bran pastes were analysed to evaluate the effects of blueberry and blackcurrant powder enrichment on the physicochemical and nutritional properties of oat bran paste. The cellular study on HepG2 cell model was conducted to evaluate the anticancer properties of the blueberry and blackcurrant powder enriched oat bran pastes.

Answers to the hypotheses

1. Enrichment with blueberry or blackcurrant powder increased the contents of phenolic compounds and enhanced the antioxidant capacity of the oat bran paste.
2. The phenolic compounds from the extracts of blueberry and blackcurrant powder reduced the reducing sugar released from oat bran paste during the *in vitro* digestion, and this, in part, was due to the α -amylase and α -glucosidase inhibitory activities of blueberry and blackcurrant enriched oat bran pastes.
3. The extracts from blueberry and blackcurrant enriched oat bran paste exerted anticancer properties towards HepG2 cells via induction of the cell apoptosis, as well as inhibition of the cell proliferation and cell invasion of HepG2 cells.

9.2 General discussion of the thesis

Numerous studies have reported the health promoting potential of bioactive compounds from berry fruits (Battino *et al.*, 2009; Karasawa & Mohan, 2018; Szajdek & Borowska, 2008). The consumption of a combination of berries and fibre-rich cereals may reap benefits on multiple levels, and which requires investigation of their nutritional attributes for the specific physiological effects (Dhingra, Michael, Rajput, & Patil, 2012). The results in this thesis provide novel insights into their possible mechanisms. This research aimed to investigate the effects of enriching oat bran paste with the bioactive compounds from blueberry and blackcurrant powders, specifically in terms of effects on antioxidant, anti-diabetic, and anticancer properties *in vitro*, chemical and in a cell line model.

Berries have a positive image and their inclusion in cereal products appears to be a promising strategy to improve their physicochemical characteristics (**chapter 4**). Addition of blackcurrant or blueberry powder in oat bran increased the final viscosity of the paste, which could be due to the effect of the soluble dietary fibre (β -glucan) on the starch content of the pastes, resulting in the formation of an entanglement of molecules during the pasting process. The pigment of the blueberry and blackcurrant powders significantly increased the redness of the paste colour, while the sugars and amino acids reaction enhanced the darkness of the paste colour during the pasting.

An imbalance between pro-oxidants and antioxidants is one of the primary pathogenic mechanisms to the progression of obesity and cancer. Bioactive compounds from berry fruits can contribute to physiological functions and act as antioxidants to protect human bodies (Lobo, Patil, Phatak, & Chandra, 2010; Salehi *et al.*, 2020). Enrichment of oat bran with blackcurrant or blueberry powder increased the total phenolic compounds and the antioxidant capacity in undigested extracts as well as digested extracts, when compared with

oat bran paste (**chapter 5**). This was in agreement with hypothesis 1 of this thesis. Gallic acid, protocatechuic acid, hydrobenzoic, and syringic acid were the most abundant phenolic acids in blueberry and blackcurrant enriched oat bran pastes (**chapter 4**), and they were identified as being potent antioxidant compounds.

A high glycaemic load is caused by high levels of starch degradation, and these have been shown to have positive correlations with the risk of T2DM (Eleazu, 2016). Oat bran has been illustrated to have positive effects on postprandial glucose levels and body mass index, which might be regulated by the dietary fibre in the oat bran (Ulmius *et al.*, 2011). This study revealed that the combination of berry fruit and fibre reduced the glycaemic response (**chapter 4**). A greater control of reducing sugar released was shown in blueberry and blackcurrant powder enriched oat bran paste than in the pure oat bran paste. This was consistent with the hypotheses 2 in this thesis. This potential health benefit is affected by the food matrix and bioactive components present in foods that positively influence postprandial glycaemia, metabolic and endocrine responses. Oat bran paste enriched with blueberry or blackcurrant powder had a significantly increased soluble dietary fibre content, that may form a non-starchy network of fibre and protein, which could impair the enzyme accessibility.

Granular starch digestion includes two steps, firstly binding of the α -amylase with the starch granules, then the catalytic hydrolysis of starch by α -glucosidase (Butterworth, Warren, & Ellis, 2011; Dhital, Lin, Hamaker, Gidley, & Muniandy, 2013). Phenolic compounds from berry fruits have been shown to delay the hydrolysis process of starch (Hanhineva *et al.*, 2010; Lin *et al.*, 2016). Therefore, the effects of the polyphenols on the binding of α -amylase and α -glucosidase with starch granules were also studied in this thesis (**chapters 6 & 7**). Cyanidins, delphinids, and malvidins in the extracts of blueberry and blackcurrant powders inhibited the activity of human saliva α -amylase and α -glucosidase showing the potential to retard starch digestion. This was in agreement with hypothesis 2 of this thesis, suggesting that the phenolic

compounds from the extracts of blueberry and blackcurrant powder exhibited the α -amylase and α -glucosidase inhibitory properties. Through enzyme kinetics analysis, blueberry and blackcurrant enriched pastes were found to exhibit mixed inhibition towards α -amylase, and competitive inhibition towards α -glucosidase. The positive linear relationships between IC₅₀ and K_{ic}, and between IC₅₀ and K_{iu} suggested that the inhibition activities of the extracts from these pastes can be attributed to the binding of anthocyanidins with these two digestive enzymes. The molecular docking study on the binding four major anthocyanidins from blueberry and blackcurrant powders with α -amylase and α -glucosidase further confirmed that the anthocyanidins promoted the binding of α -amylase and α -glucosidase with substrates, forming the “inhibitor-substrate-enzyme” or “inhibitor-enzyme” complexes. The results in **chapter 6 & 7** suggest that functional molecules other than fibre, particularly the phenolic compounds, may have an impact on the enzymatic digestion, and that the phenolic compounds may act in a synergistic role.

Berry fruits and cereal foods are being increasingly investigated for their potential anticancer properties. Anthocyanins, flavonoids, or other antioxidants, seem to offer a means to reduce the risk of cancers as evidenced by numerous *in vivo* and clinical studies (Baby, Antony, & Vijayan, 2018; Banerjee & Rajamani, 2013). The mechanistic details of this potential remain somewhat elusive even though certain pathways have been discovered. These pathways are responsible for the modulation of different cellular processes, showing certain common signalling events, such as arrest of cell cycle, inhibition of cyclins, induction of apoptosis via activation of caspases and regulation the protein expressions of Bcl-2 family members. **Chapter 8** reports the anticancer potential of the extracts from blueberry and blackcurrant enriched pastes against the HepG2 cell model. The results showed that the bioactive compounds from the extracts of blueberry and blackcurrant enriched pastes exerted the anticancer properties via regulating the expression of apoptotic-related proteins including Bcl-

2 and Bax, and further modulate the expression of Caspase-3. This was consistent with the hypothesis 3 of this thesis, suggesting that the anticancer property of the blueberry and blackcurrant powder enriched oat bran pastes is involved in the cell apoptosis of HepG2. Several common constituents in berry fruits such as quercetin, kaempferol and pterostilbene have been found to attenuate reactive oxygen species in HepG2 cells via the Nrf2/HO1 antioxidant response element signalling pathway (Saw *et al.*, 2014), suggesting that the induction of antioxidant defence is one of the potential mechanisms via which the extracts from blueberry and blackcurrant enriched pastes provide anticancer properties.

9.3 Future work

Evaluation of the mechanistic details were undertaken via chemical and cell model in this thesis. Future work could be directed to develop a deeper analysis of the particular mechanisms, and new techniques linking with *in vivo* studies. There are some ideas that I would try regarding the antidiabetic and the anticancer activities of the extracts of blueberry and blackcurrant enriched oat bran paste using a mouse model:

1. A high-fat-induced diabetic mouse model would be employed to evaluate the antidiabetic activities of the extracts of blueberry and blackcurrant enriched oat bran paste. The body weight, blood glucose, serum lipid profiles and insulin tolerance would be measured after the administration of different concentrations of these extracts (for example: 50, 100, and 200 mg/kg). Signalling pathways related to the insulin resistance of pancreatic β -cell can be analysed, and the expressions of the protein molecules in these pathways would be tested.
2. It would be interesting to determine whether the extracts of blueberry and blackcurrant enriched pastes could be used in the clinic for the treatment of liver cancer. The therapeutic effects of these extracts could then be assessed using

diethylenetriamine stimulated mice, and the potential mechanisms of extracts against liver cancer could also be explored (such as suppression of NF- κ B activities).

9.4 General conclusion of this thesis

The general conclusion of this thesis suggests that the consumption of oat bran enriched with berry fruits may have the synergic effects in terms of antioxidant, antidiabetic and anticancer activities, which are potentially due to the interaction of the phenolic compounds from blueberry and blackcurrant power with the other food components present in this food matrix. This information could be used to design blueberry or blueberry enriched cereal food products or formulated into drug-like treatments for T2DM and cancers.

Appendix

A.1 The information of chemicals and antibodies

All Chemicals were bought from the companies list in **Table A-1** and were of analytical grade.

Table A-1 Information of chemicals and antibodies

Chemicals	Source	Identifier
2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)	Sigma-Aldrich	Cat# A1888
2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ)	Sigma-Aldrich	Cat# 93285
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich	Cat# D9132
Gallic acid	Sigma-Aldrich	Cat# G7384
3,5-Dinitrosalicylic acid, 98%, ACROS Organics™ (DNS)	Sigma-Aldrich	Cat# AC156441000
Folin & Ciocalteu's phenol reagent	Sigma - Aldrich	Cat# F9252
Pepsin (EC 3.4.23.1)	Fisher Scientific	Cat# P/1120/53
Pancreatin (EC 232-468-9)	AppliChem	Cat# A0585
α -amylglucosidase (<i>Aspergillus Niger</i>)	Megazyme	Cat# E-AMGDF
α -amylase (Human saliva, EC 3.2.1.1)	Sigma-Aldrich	Cat# A1031
α -glucosidase (<i>Saccharomyces cerevisiae</i> , EC 3.2.1.20)	Sigma-Aldrich	Cat# G5003
p-nitrophenyl- α -glucopyranoside (p-NPG)	Sigma-Aldrich	Cat# N1627
Invertase (EC 3.2.1.26)	Sigma-Aldrich	Cat# I0408
Acarbose	Sigma-Aldrich	Cat# A8980
Celite	Megazyme	Cat# NO. G-CELITE

To be continued (**Table A-1**)

Chemicals	Source	Identifier
2-(Nmorpholino) ethanesulfonic acid (MES)	Megazyme	Cat# NO. B-MES250
tris(hydroxymethyl)aminomethane (TRIS)	Megazyme	Cat# NO. B-TRIS500
2,7 dichlorodihydrofluorescein-diacetate (DCFH-DA)	Sigma-Aldrich	Cat# D6883
Antibodies	Companies	identifier
Bcl-2	Beyotime	Cat# AB112
Bax	Beyotime	Cat# AF1270
Caspase-3	Beyotime	Cat# AF1213
Nrf-2	Beyotime	Cat# AF1609
HO1	Beyotime	Cat# AF1333
GAPDH	Beyotime	Cat# AF1186

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